THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 12, Issue of March 24, pp. 6440–6449, 1995
Printed in U.S.A.

Rapid Identification of Highly Active and Selective Substrates for Stromelysin and Matrilysin Using Bacteriophage Peptide Display Libraries*

(Received for publication, December 1, 1994, and in revised form, January 9, 1995)

Matthew M. Smith, Lihong Shi, and Marc Navre‡

From the Department of Biochemistry, Affymax Research Institute, Santa Clara, California 95051

The discovery of useful peptide substrates for proteases that recognize many amino acids in their active sites is often a slow process due to the lack of initial substrate data and the expense of analyzing large numbers of peptide substrates. To overcome these obstacles, we have made use of bacteriophage peptide display libraries. We prepared a random hexamer library in the fd-derived vector fAFF-1 and included a "tether" sequence that could be recognized by monoclonal antibodies. We chose the matrix metalloproteinases stromelysin and matrilysin as the targets for our studies, as they are known to require at least 6 amino acids in a peptide substrate for cleavage. The phage library was treated in solution with protease and cleaved phage separated from uncleaved phage using a mixture of tether-binding monoclonal antibodies and Protein A-bearing cells followed by precipitation. Clones were screened by the use of a rapid screening assay that identified phage encoding peptide sequences susceptible to cleavage by the enzymes. The nucleotide sequence of the random hexamer region of 43 such clones was determined for stromelysin and 23 for matrilysin. Synthetic peptides were prepared whose sequences were based on some of the positive clones, as well as consensus sequences built from the positive clones. Many of the peptides have k_{cat}/K_M values as good or better than those of previously reported substrates, and in fact, we were able to produce stromelysin and matrilysin substrates that are both the most active *and* smallest reported to date. In addition, the phage data predicted selectivity in the P_2 and P'_1 positions of the two enzymes that were supported by the kinetic analysis of the peptides. This work demonstrates that the phage selection techniques enable the rapid identification of highly active and selective protease substrates without making any *a priori* assumptions about the specificity or the "physiological substrate" of the protease under study.

The selectivity of a protease is dictated, in part, by the sequence of amino acids it recognizes in its active site before cleaving the substrate. From a kinetic point of view, the higher the value of the specificity constant k_{cat}/K_M (1), the better a peptide is as a substrate for that protease. Highly selective proteases will have high k_{cat}/K_M values for only a small number of peptides, whereas a larger number of peptides will have similar k_{cat}/K_M values for broad specificity proteases. The identification of the optimized peptide substrates is thus an important part of protease characterization. A time consuming step in protease characterization, however, is finding an optimal substrate. In the case of enzymes like the matrix metalloprotease (MMP),[1] fibroblast collagenase, or the protease of the human immunodeficiency virus (HIV protease), a peptide substrate can be prepared based on the cleavage site of the physiological substrate collagen (2) or the HIV polyprotein (3, 4), respectively. These peptides can be dramatically improved by substitution of individual residues with other amino acids (5–7), so that the original substrate-derived peptide may not represent the optimal substrate. In some cases a true physiological substrate is unknown. This was true in the case of the MMP stromelysin. Investigators (8) found obtaining good peptide substrates so frustrating that they randomly screened commercially available peptides for active compounds. Even when substrate information is available, investigators (9, 10) have used proteins as substrates to further assess the sequence specificity of the protease under study.

To overcome these problems, we have made use of filamentous bacteriophage-based peptide display libraries to find optimal substrates. Phage display libraries have been used with great success to find epitopes for monoclonal antibodies and to improve the affinity of peptides for receptors (11, 12). Recently Matthews and Wells (13) have presented a method for the use of monovalent "substrate phage" libraries for discovering peptide substrates for proteases. These investigators screened a random pentamer library and isolated clones carrying substrates for Factor X and a mutant form of subtilisin. However, these sequences were not tested as solution phase peptide substrates, and hence the predictive nature of the method was not evaluated. We have been developing an analogous method using polyvalent phage. The approaches presented here have enabled us to screen a greater number of phage (and thus larger libraries), characterize putative substrate clones more quickly, and to generate consensus sequences from these hits. The peptides prepared based on our screen are as good or better substrates than literature standards for the protease being examined.

To critically assess the method, we have chosen the MMPs stromelysin and matrilysin as the focus of our studies. The MMPs represent a family of enzymes that recognize at least 6 amino acids in their subsites, as shown by the sensitivity of

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: 3410 Central Expressway, Santa Clara, CA 95051. Tel.: 408-522-5718; Fax: 408-481-0522; Internet: Marc_Navre@qmgates.affymax.com.

[1] The abbreviations used are: MMP, matrix metalloprotease; HIV, human immunodeficiency virus; mAb, monoclonal antibody; ON, oligonucleotides; Kan^R, kanamycin resistance; tu, transducing units; fTC, fAFF1-tetherC; fTC-Good, fTC carrying a good substrate for stromelysin; fTC-Bad, fTC carrying a bad substrate for stromelysin; fTC-LIB, fTC derivative used for preparing the library; DPA, 3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Ac, acetyl; sfSTR, COOH terminally truncated form of stromelysin; pro-sfSTR, the proenzyme form of sfSTR.

k_{cat}/K_M to the substitution of amino acids in positions P_3 to P'_3 (2, 5, 14–17). In addition, when this work was initiated, only a limited amount of information had been available for stromelysin (8, 18) or matrilysin (16). Using recombinant forms of these enzymes, we have screened a random hexamer library and have used the sequence information from positive clones to prepare new, highly active peptide substrates. The best of these peptides are the most active stromelysin and matrilysin substrates reported to date. In addition, previous investigators have always used peptides no smaller than heptamers in the concern that shorter compounds would not be sufficiently active. With the availability of optimized amino acids at each position, we have demonstrated the opposite: that hexapeptides are superior MMP substrates, a finding not expected at the outset of this study. Finally, the phage data successfully predicted ways in which these substrates could be made selective toward matrilysin or stromelysin.

Most importantly, we have demonstrated that we can identify protease substrates without making any *a priori* assumptions about the specificity or the "physiological substrate" of the enzyme. This approach will be valuable for the study of proteases where peptide substrates are unavailable and sites of cleavage *in vivo* are unknown.

MATERIALS AND METHODS

Reagents—Library competent MC1061 (F^-) *Escherichia coli* and nitrocellulose were from Bio-Rad. Pansorbin (Protein A-bearing *Staphylococcus aureus*) cells were obtained from Calbiochem. Polyvinylidene fluoride membranes were from Millipore, Inc. K91 (F^+) and MC1061 (F^-) strains of *E. coli* (19) were obtained from Steve Cwirla of Affymax. mAb 179 recognizes an epitope (ACLEPYTACD) of the human placental alkaline phosphatase protein with subnanomolar affinity.[2] mAb 3-E7 (20) was from Gramsch Laboratories (Schwabhausen, Germany). Stromelysin was expressed as a His_6-NH_2 terminally tagged, COOH terminally truncated proenzyme (pro-sfSTR) in a soluble form in *E. coli* and purified with some minor modifications[3] of the protocol of Marcy *et al.* (37). A His_6-NH_2 terminally tagged form of matrilysin was also expressed in *E. coli*, and after recovery from inclusion bodies was purified by nickel-chelation chromatography, refolded, and further purified by Blue-Sepharose chromatography.[3] Purified activated enzymes were prepared with trypsin, treated with an excess of soybean trypsin inhibitor and stored at −80 °C.

Construction of Vectors and Phage Libraries—fAFF1-tether C (fTC) was constructed by inserting oligonucleotides (ON) 1200/1201 (5′-CTCCCACTCCTACGGAGGATTCTTAGGTGCATGCCTGGAACCGTACACCGCTTGCGACGTAGGCCTGGTACCGGAATTCGCTTGT-3′ and 5′-GCGAATTCCGGTACCAGGCCTACGTCGCAAGCGGTGTACGGTTCCAGG CATGCACCTAAGAATCCTCCGTAGGAGTGGGAGTAGA-3′; see Fig. 2*A*) into the *Bst*XI sites of fAFF1 (19). Control substrate phage fTC-Good and fTC-Bad were constructed by inserting ON1280/1281 (5′-CGGTGGTGGTAGTCCGCTAGCCCTGTGGGCTGTAC-3′ and complement) (Good substrate control; Fig. 2*B*) or ON1282/1283 (5′-CGGTGGTGGTAGTAACCCGGTTGAACCAGCTGTAC-3′ and complement) (Bad substrate control; Fig. 2*B*) into *StuI/Kpn*I-cut fAFF-tether C. fAFF-TC-Good-Kan^R (Kan^R = kanamycin resistance) was constructed by inserting the T4 DNA polymerase-treated 1.4-kilobase *Ava*I fragment containing the Kanamycin resistance gene from pCR1000c (Invitrogen) inserted in a reverse orientation between the T4 DNA polymerase-treated *Ava*I and *Nco*I sites of fTC-Good. fTC-LIB was constructed from fAFF-tether C by inserting oligonucleotides ON1526/1527 (5′-TCTGGAACCGTACACCGCATGCGACTCGAGCGAGACCGAAGACGTACTGGTAC-3′ and complement) into the *SphI/Kpn*I sites of fAFF-tether C. The fAFF-TC-LIB-N_6 library was constructed by cloning degenerate oligonucleotides (5′-CAGMNNMNNMNNMNNMNNMNNACCACTACCACCGC-3′, where N is A, C, G, T (equimolar) and M is C or A (equimolar)) annealed with an 18 inosine-containing complementary oligonucleotide (ON1439/1440; see Fig. 2*C*) into *KpnI/Xho*I-cut fAFF-TC-LIB at a 5:1 oligo/vector molar ratio and electroporating into *E. coli* MC1061 (F-).

Phage Selection—2×10^{10} phage (20 μl of the fTC-LIB-N6 library) in TCB (20 mM Tris-HCl, pH 7.4, 5 mM $CaCl_2$, 0.05% Brij-35) in a 250-μl reaction were digested with an empirically determined amount of enzyme for 1 h at 37 °C. The reaction was stopped by adding EDTA to 5 mM and then followed by the addition of bovine serum albumin to 0.1%, 100 μg of mAb 179 and 10 μg of mAb3-E7. After 30 min on ice, 100 μl of Pansorbin cells were added, and the reaction was rotated at 4 °C for 1 h. The mixture was microfuged for 2 min and the supernatant recovered to repeat the Pansorbin adsorption. The final supernatant was amplified overnight in *E. coli* K91 cells. A small aliquot of the final supernatant solution was also used for titering on K91. Clones were selected from the titer plates and grown in 2-ml cultures for dot-blot analysis.

Phage Proteolysis Assay—To precipitate phage, 20 μl of 20% polyethylene glycol, 2.5 M NaCl was added to 100 μl of phage supernatant. After incubating on ice for 30 min, the precipitated phage were microfuged for 5 min. The supernatant was aspirated and the phage resuspended in 10 μl of TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl). The phage were then distributed to wells of a flexible microtiter plate (Falcon). 90 μl of protease/buffer mix (90 μl 1.1 × TCB, 0.3 μl of enzyme) were added and the plate incubated for the appropriate time period at 37 °C. At various time points, 30-μl samples were removed and added to 70 μl of TBS + EDTA (to 5 mM final concentration) to stop the reaction. The samples were spotted onto a nitrocellulose filter with a dot-blotter (Bio-Rad) and the filter blocked with 5% non-fat milk in TBS-T (TBS + 0.05% Tween 20) for 30 min to 1 h. The filters were washed three times with TBS-T and then incubated for 1 h with a mAb 179 at 1.9 μg/ml. The washes are repeated and the filters probed with a 1:5000 dilution of goat anti-mouse IgG horseradish peroxidase-conjugate. After 1 h, the washes were repeated and the filter stained as directed using the Amersham Western Enhanced Chemiluminescence Kit.

Kinetic Analysis of Peptides—Synthetic substrates were prepared as peptides blocked at their amino termini by an acetyl group. Peptide 12 (2,4-dinitrophenyl-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2) (14) was a gift of Dr. Robert Gray (University of Louisville) and 11 (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-(3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl)-Ala-Arg-NH_2) (21) was from Bachem. Peptides at various concentrations were treated with 20–50 nM protease at 37 °C for various times. The initial rates of hydrolysis were determined by measuring the rate of formation of free amino groups using fluorescamine (2). Kinetic parameters were derived by fitting the data to the equation $v = (V_{max} \cdot [S])/(K_M + [S])$ by non-linear regression analysis (Enzfitter), and k_{cat} was determined by dividing V_{max} by the concentration of enzyme used. The sites of hydrolysis of selected substrates were determined by fast atom bombardment mass spectrometry of the high performance liquid chromatography purified cleavage products.

RESULTS

Phage peptide display vectors have been used successfully to identify peptides that bind to proteins such as antibodies. In contrast, the goal of this work is to use this methodology to identify peptides that are efficiently *cleaved* by a specific protease. In the most commonly used phage display format, the random peptide sequence is placed at or near the NH_2 terminus of the display protein, generally pIII (19, 22–25). In the method described here, we have added an additional functional group to the NH_2 terminus of pIII, a peptide "tether." The tether is a peptide sequence that enables attachment of the phage through binding of the tether sequence to an immobile phase: the simplest example being a peptide epitope tether bound to a monoclonal antibody on an agarose bead. The strategy of the selection is to subject a population of tether phage to the protease in solution, and then separate the cleaved (substrate) from the uncleaved (non-substrate) phage by capturing the undigested phage with a tether-binding resin. A schematic of a variation of this approach, used in this work, is shown in Fig. 1.

We have designed and prepared the vector fAFF-tether C (fTC; see "Materials and Methods") from the fd-derived plasmid fAFF-1 (19). The salient features of this phage, shown in Figs. 1 and 2 include the (i) target region, which can consist of random amino acids, or predetermined sequences (*i.e.* GOOD and BAD, Fig. 2, *B* and *C*) for use as positive or negative controls. The design of the control sequences is described be-

[2] R. Barrett, manuscript in preparation.
[3] R. J. Armstrong, L. Shi, and M. Navre, unpublished results.

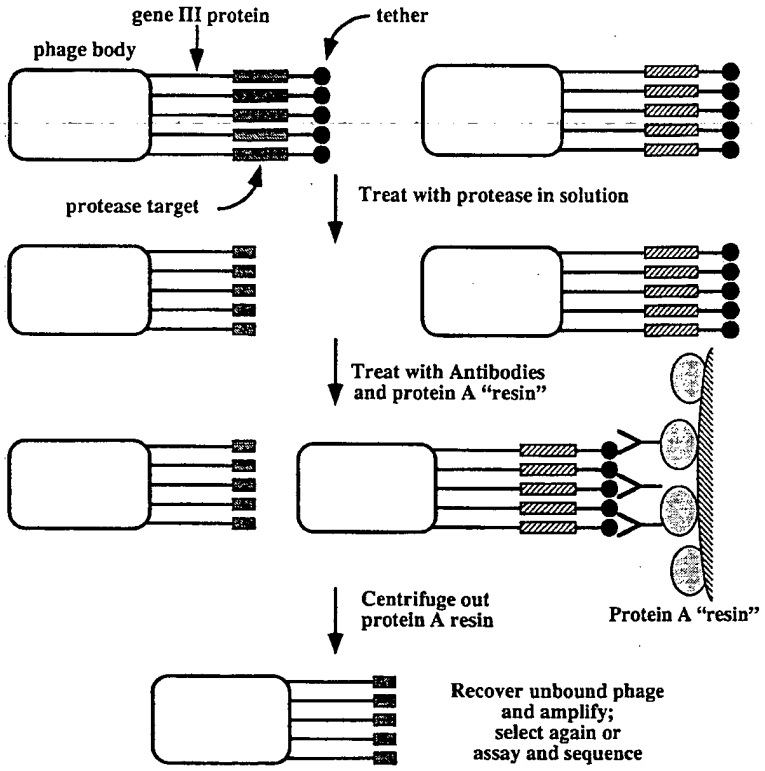

FIG. 1. **Outline of the tether phage selection.** Shown are diagrams of the fTC phage (not drawn to scale for clarity). The *gene III protein* extends from phage body from the COOH to NH_2 terminus. At the NH_2 termini are the peptide *tether* and the *protease target* domain. The phage are treated in solution with the protease. Phage carrying substrate sequences (*left side*) are cleaved in the target domain, whereas others (*right*) are not. The entire digest is treated with antibodies to the tether(s) and captured using a *resin* that carries protein A. The protein A-antibody-phage complexes are precipitated by centrifugation, and the phage that are not bound by the antibodies remain in solution and can be recovered for amplification.

A) fAFF-tether C

```
                    SphI                          StuI        EcoRI
tacggaggattcttaggtGCATGCctggaaccgtacaccgcttgcgacgtAGGCCTggtaccgGAATTCgcttgt
 Y  G  G  F  L  G  A  C  L  E  P  Y  T  A  C  D  V  G  L  V  P  E  F  A  C
```

B) fAFF-tether C-GOOD

```
tgc gac gta ggc ggt ggt ggt agt ccg cta gcc ctg tgg gct gta ccg gaa
 C   D   V   G   G   G   G   S   P   L   A   L   W   A   V   P   E
```

fAFF-tether C-BAD

```
tgc gac gta ggc ggt ggt ggt agt aac ccg gtt gaa cca gct gta ccg gaa
 C   D   V   G   G   G   G   S   N   P   V   E   P   A   V   P   E
```

C) fAFF-tether C-LIBRARY

```
TGC GAC TCG AGC GGT GGT AGT GGT III III III III III III CTG GTA CCG GAA
ACG CTG AGC TCG CCA CCA TCA CCA NNM NNM NNM NNM NNM NNM GAC CAT GGC CTT
 C   D   S   S   G   G   S   G   X   X   X   X   X   X   L   V   P   E
```

FIG. 2. **Construction of fTC and its derivatives.** *A*, fAFF-tether C: the oligo pair 1200/1201 (see "Materials and Methods") were hybridized and ligated into *Bst*XI fAFF-1 (19). The *lower line* shows the translated peptide sequence of the pIII protein starting from the predicted site of signal peptide cleavage, leaving YGGFL at the NH_2 terminus of the phage (19). The *underlined* peptide sequence (ACLEPYTACD) is the epitope for mAb 179.[2] *B*, sequence of inserts of fTC-Good and Bad. These clones were derived from fTC as described under "Materials and Methods." The sequences shown start at the end of the mAb 179 epitope (ACLEPYTACD). *C*, construction of the library. fTC-LIB (see "Materials and Methods") was cleaved and ligated with the degenerate oligo pair 1439/1440 (*I* indicates inosines, which are expected to hybridized to all bases (24) and M = A or C). The resultant library is shown, where *X* indicates any amino acid or stop codon. The sequences shown start at the end of the mAb 179 epitope (ACLEPYTACD) as in *B*.

low; (ii) the tether region. We have employed a dual tether design, in which the tether consists of the epitopes for the anti-dynorphin mAb 3-E7 (YGGFL) (19, 20) and the mAb 179 epitope ACLEPYTACD (see Fig. 2, *A* and *C*).

A requirement of this approach is that the phage molecule be cleaved only in the "target" random peptide region. Although this is anticipated, as filamentous phage are generally viewed as being protease resistant (26), we chose to test this proposal. We prepared two phage clones in fTC, one carrying a good substrate (fTC-Good) sequence for the MMP stromelysin, and one carrying a poor substrate (fTC-Bad) (see "Materials and Methods" and Fig. 1). We were faced with two choices of known substrate sequences for stromelysin: those based on substance P (8, 17) or a sequence based on collagenase cleavage sites (18). We chose to build a generic MMP good substrate, Pro-Leu-Ala-Leu-Trp-Ala, based on the results of Netzel-Arnett *et al.* (16) that was suitable not only for stromelysin, but matrilysin as well. As shown later, this peptide has a k_{cat}/K_M value for sfSTR comparable with substance P (8) and a 2,4-dinitrophenyl-octapeptide fluorogenic substrate (18). The control phage were incubated with sfSTR at various times for up to 1 h. The digests were then analyzed by immunoblot analysis using the anti-tether mAb 179. As shown in Fig. 3*A*, after exposure to sfSTR, the pIII protein of fTC-Bad was essentially undisturbed, whereas the pIII of fTC-Good lost its tether. This indicates that only the target sequence of the pIII protein was specifically

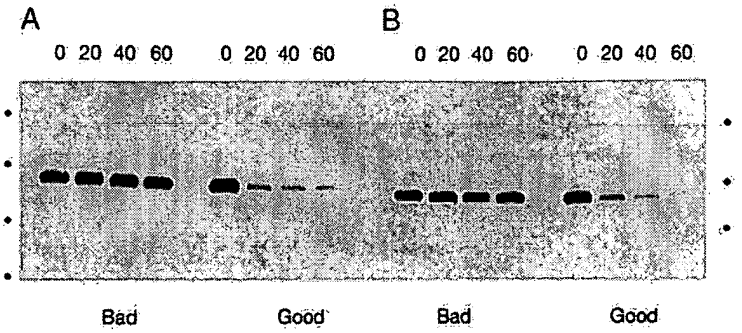

FIG. 3. **Immunoblot analysis of phage clones treated with protease.** sfSTR (at 5 μg/ml, *panel A*) or matrilysin (at 5 ng/ml, *panel B*) were incubated with fTC-Good or fTC-Bad (*Good* or *Bad*) at 5×10^9 tu/ml (approximately 10 pM phage) at 37 °C, and aliquots removed at 0, 20, 40, and 60 min and added to 5 μl of SDS gel buffer and boiled to quench the reaction. The samples were subjected to electrophoresis through 12% polyacrylamide gels containing SDS and transferred to nitrocellulose, which was probed with mAb 179 and detected as described under "Materials and Methods." The positions of the protein standards on the gels are indicated by *dots* and are M_r 106, 80, 49.5, and 32.5 (*panel A* only) $\times 10^3$.

cleaved by sfSTR, and other regions of the protein were untouched. Similar results were observed for the matrilysin (Fig. 3*B*), tissue plasminogen activator, and HIV protease (using appropriate positive controls; data not shown). In addition, titering of phage (fTC-Good and fTC-Bad) before and after digest showed no effect of proteolysis (with any of the proteases tested) on infectivity (data not shown).

Test with Mock Library—To allow us to easily distinguish fTC-Good and fTC-Bad, the tetracycline resistance gene in fTC-Good was replaced with a kanamycin resistance marker (Kan^R; see "Materials and Methods"). A mock library for testing our selection procedure was prepared by spiking 10^9 fTC-Bad phage (in 100 μl) with 10^5 fTC-Good-Kan^R phage. The mock library was then treated with 1 μg/ml sfSTR for 1 h at 37 °C. The reaction was quenched with EDTA and subjected to one round of the selection procedure described under "Materials and Methods." Various dilutions of the final supernatant solution were titered on both tetracycline (fTC-Bad) and kanamycin (fTC-Good-Kan^R). In many repetitions of this experiment, the recovery of fTC-Good was always nearly quantitative, whereas there was generally a 100-1000-fold loss of fTC-Bad, depending on experimental conditions. This result suggests that a single round of selection could enrich a rare good substrate over a large background of poor substrate. Of note is the fact that we use two antibodies simultaneously in our selections. Although the method does work if we use either antibody alone, we have found that the combination of mAbs yields consistently lower backgrounds and reduces the number of clones that are unreactive to Ab179 in our screening assays (see below).

An important concept in phage display is affinity selection. This involves using limiting levels of receptor or phage proteins in order to isolate phage carrying peptides of high affinity (27, 28). The analogous approach for a protease selection would be the use of less protease in order to drive the selection toward those clones carrying better substrates (practically speaking, those cleaved at a lower or limiting protease concentrations). As a first test of this proposal, we examined recovery of good phage from the mock library at decreasing protease concentration. As shown in Fig. 4, when the mock library was treated with less sfSTR, the amount of good phage recovered decreased, and in an essentially linear fashion. This result indicates that the use of lower concentrations of proteases could be employed to gain more selectivity (that is, selection of clones that require lower protease concentrations to be cleaved). This is also a useful test for determining starting protease concentrations. By working with protease levels above that which gives ~100% recovery, more protease is being used than is needed. In contrast, use of too low a protease concentration could result in the recovery of too few clones.

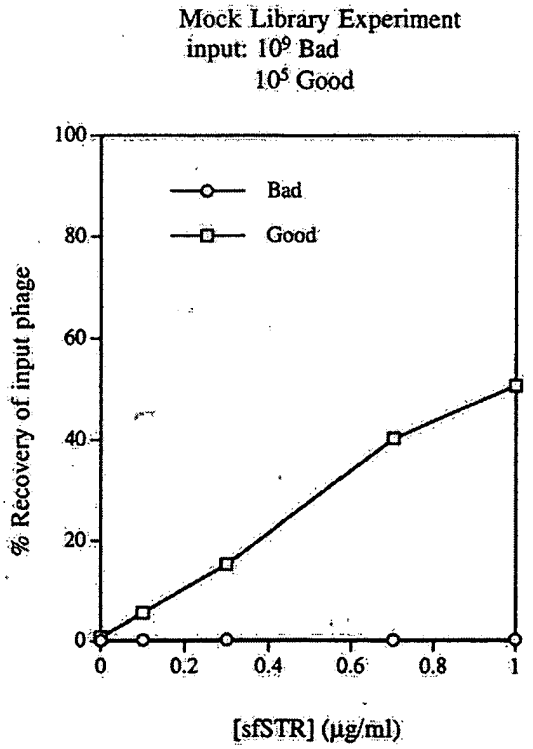

FIG. 4. **Recovery of good phage with decreasing concentrations of sfSTR.** A preparation of 10^9 fTC-Bad phage (in 100 μl) was spiked with 10^5 fTC-Good-Kan^R phage and treated with varying concentrations of sfSTR for 1 h at 37 °C. The reaction was quenched with EDTA and subjected to one round of the selection procedure described under "Materials and Methods." Various dilutions of the final supernatant solution were titered on both tetracycline and kanamycin. The percentage of recover of fTC-Bad (○) and fTC-Good-Kan^R (□).

Phage Proteolysis Assay—One purpose of the peptide display approach is to obtain as much information as possible from the peptides while they are on phage, before turning to the use of synthetically prepared substrates. We thus developed a simple and rapid method for determining whether the peptide sequence carried by an fTC clone is a good or bad substrate. We had previously determined that while phage would bind to nitrocellulose, the cleaved tether peptide would not (data not shown). Thus, by simply spotting reaction time points onto a nitrocellulose filter and probing with the anti-tether antibody, we could observe the time-dependent loss of the tether from the phage. As shown for the controls fTC-Good and fTC-Bad in Fig. 5, similar signal intensities are observed for the two phage

clones when they were not treated with sfSTR. After 10 or 60 min of protease treatment, however, the intensity of the fTC-Good spots decrease, while no such loss is seen for fTC-Bad. This dot-blot assay is thus useful as a way of monitoring the proportion of positive sequences generated during each round of screening.

Screening the Library—A library of random hexamers, generated using the DNA sequence $(NNK)_6$ (19) was prepared in fTC-LIB as described under "Materials and Methods." The library contained 2×10^8 independent recombinants. In the first round, 2×10^{11} tu (about 1000 equivalents of each clone) were treated with sfSTR, subjected to the selection protocol, and the recovered phage were amplified. After each round, 12 clones were analyzed using the phage proteolysis assay. The results of the screening are shown in Table I. In the first series of screening (rounds A1-A7), the output was not titered before the next round of screening, thus variable amounts of phage were used as input for the succeeding round. In contrast, for series C, the output phage were titered before the next round, and a constant input was used (Table I). Fig. 5 shows such an analysis for 12 clones chosen randomly from the library and after three rounds of screening in series A. There are no positive phage among the randomly chosen clones (although one is missing or unreactive with the antibody). A number of the clones isolated in round 3, however, appear to be substrates for the enzyme. In later rounds of screening at reduced protease concentrations, a number of clones were found that appeared to be non-reactive with mAb179 prior to sfSTR treatment (see Table I). Sequence analysis of four of these clones indicated that the epitope encoding sequence has not been altered, and blot analysis showed that the phage were still recognized by mAb 3-E7, indicating that the gene III protein had not been cleaved (data not shown). We believe that these non-reactive clones, which appear during more stringent selection, are due to alterations in the secondary or tertiary structure of the NH_2 terminus of the pIII protein in such a way as to mask the mAb 179 epitope.

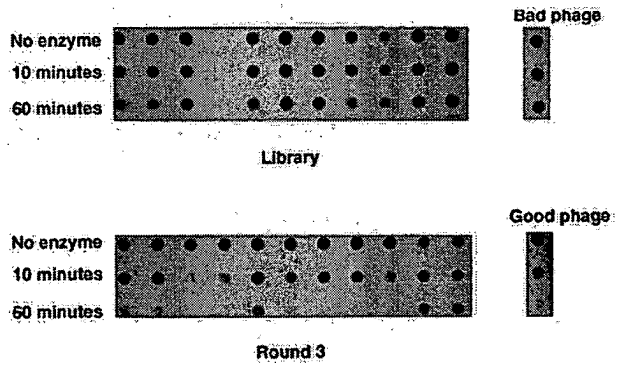

FIG. 5. **Proteolytic analysis of phage clones.** Individual clones from the initial library or round 3 of screening were prepared, digested with sfSTR for 0, 10, or 60 min, dotted onto nitrocellulose, and detected as described under "Materials and Methods." Also shown are identical treatments with fTC-Good and fTC-Bad, showing digestion of the former, but not the latter. Six clones from round three are hits based on this assay.

The nucleotide sequence of a number of positive sequences from round 3 were determined, and the deduced amino acids corresponding to the random target sequences are shown in Table II. Also shown for comparison are sequences from clones chosen randomly from the library. No pattern is apparent for the negative clones, but some patterns did appear for the positive sequences. To further characterize these sequence trends, the selection with sfSTR was pursued for three more rounds. In addition, since the majority of the clones screened were judged to be hits at this point, the concentration of sfSTR used in the selection was dropped 5-fold for round A4 and again at round A6. In addition, a new selection series (series C) was initiated, this time starting with only 2×10^{10} tu from the library, and using an initial sfSTR at 1 μg/ml (see Table I). As can be seen in Table IIA, in both the A and C series, the number of non-reactive clones increases as the selective pressure (decreasing protease activity) is increased. Positive sequences from the rounds of series A, as well as those from the most recent selection series (C), are also shown in Table IIA.

The trends from the 43 sequences in Table II are summarized in Fig. 6. Most invariant was the B position, where the bulk of clones carried a Pro. In all MMP substrates examined thus far, Pro appears to be favored in the P_3 position (2, 5, 14–17), suggesting that we should "lock in" the B position as being equivalent to P_3 (this was confirmed by later analysis of the cleaved peptides; see Table III). Looking at trends in other positions, we found stromelysin favored large hydrophobic groups (Leu, Met, Phe, and Tyr) in positions C (P_2), Glu, or Ala in D (P_1), and Leu or Met in E (P'_1). Positions A and F were not as selective: Ala and Val predominated at A (P_4), whereas hydroxy (Ser and Thr) and small aliphatic residues were favored at F (P'_2). Since the protease seemed to recognize the N_6 target site of the library as P_4-P'_2 sites, we have little information on trends in the P'_3 site.

Testing Positive Sequences as Peptides—To verify that the sequences generated were indeed substrates for stromelysin,

TABLE I

Summary of data from two sets of protease selection

For each round of selection, the antibody used (Abs), the number of transforming units of phage used (phage input), concentration of sfSTR used [sfSTR], the number of clones screened by phage proteolysis assay, and the number and percentage that were positive (hits) or non-reactive are shown. The concentration of stromelysin used in the phage proteolysis for a given round was the same used in the selection protocol.

Selection statistics				Data from phage proteolysis assays				
Round	Phage input	Screening Ab(s)	[sfSTR]	Screened	No. hits	% hits	No. non-reactive	% non-reactive
			$\mu g/ml$					
A1	2×10^{11}	179	5	12	2	17	0	0
A2	4×10^{10}	179	5	12	10	84	0	0
A3	5×10^{8}	179	5	12	10	84	0	0
A4	9×10^{9}	179	1	12	8	67	0	0
A5	5×10^{11}	179	1	12	9	75	1	8
A6	9×10^{10}	179	0.2	12	4	33	5	42
A7	2×10^{11}	179	0.2	12	1	8	11	92
C1	2×10^{10}	179 + 3-E7	1	12	2	17	3	25
C2	2×10^{10}	179 + 3-E7	1	12	8	67	2	17
C3	2×10^{10}	179 + 3-E7	1	12	10	83	1	8.3
C4	2×10^{10}	179 + 3-E7	1	120	47	40	54	45

TABLE II

Translated sequence of peptides found in phage clones chosen from the sfSTR screen (A), the matrilysin screen (B), or randomly from the library (C)

The sequence corresponding to the random region of the library (oligonucleotides NNK_6) was translated, and is shown according to position. Clone no. indicates the source of the clone: A3–4, for example, comes from round 3 of session A, and was the fourth clone analyzed. Score indicates phage strong cleavage by the protease (++) or not at all (−) in the phage proteolysis assay (see Fig. 3). A-F represent the six positions in the N_6 random target region of the clones from the fTC library. A number of clones (*i.e.* A6–1, C–33, E3–71) have been aligned in order to place their prolines in the B position.

	Clone no.	Score	A	B	C	D	E	F	
A	A3–3, A6–5	++	R	P	H	Q	V	V	
	A3–4	++	L	P	F	N	M	T	
	A3–7	++	V	P	R	A	I	S	
	A3–8	++	E	A	F	G	M	R	
	A3–9	++	R	P	Q	T	L	A	
	A4–3	++	G	P	M	A	M	T	
	A4–9	++	A	P	Q	A	T	V	
	A4–11	++	A	P	S	A	M	S	
	A5–2	++	I	P	F	E	Q	R	
	A6–1	++		P	F	N	S	V	S
	A6–6	++	K	A	F	E	L	T	
	A6–9	++	S	L	Y	E	L	R	
	C4–1	++	G	A	N	F	F	E	
	C4–3	++	N	D	Y	P	A	F	
	C4–4	++	G	V	D	F	L	E	
	C4–5	++	F	N	E	F	S	N	
	C4–6	++	R	P	E	S	M	T	
	C4–8	++	A	P	L	Q	S	T	
	C4–10	++	I	P	S	E	M	R	
	C4–12	++	K	P	H	A	L	S	
	C4–16	++	A	P	L	E	S	I	
	C4–19	++	A	P	L	E	A	V	
	C4–29	++	A	P	L	T	L	S	
	C4–36	++	E	P	M	S	L	S	
	C4–39	++	V	P	M	S	M	S	
	C4–33	++		P	K	P	L	A	L
	C4–34	++		P	M	A	M	M	G
	C4–35	++	A	P	F	R	L	E	
	C4–38	++	V	P	L	E	T	R	
	C4–44	++	V	P	M	S	M	S	
	C4–50	++	G	P	Y	S	L	T	
	C4–58	++	V	P	M	E	I	K	
	C4–60	++	A	W	L	L	R	W	
	C4–77	++	E	P	Y	E	L	Y	
	C4–85	++	T	P	F	A	F	A	
	C4–94	++	V	P	Y	E	L	A	
	C4–95	++	E	P	L	G	L	V	
	C4–96	++	A	P	Y	E	V	M	
	C4–97	++	M	L	L	A	S	T	
	C4–105	++	G	P	W	E	S	K	
	C4–106	++		P	M	E	M	V	E
	C4–107	++	V	P	L	E	L	K	
	C4–117	++		P	M	A	M	V	A
B	E3–1	++	M	P	L	S	L	T	
	E3–2, 43	++		P	L	E	I	R	A
	E3–8	++	H	P	M	D	V	K	
	E3–9	++	M	L	A	E	L	R	
	E3–14	++	I	L	E	L	Q	G	
	E3–26	++	A	P	M	Q	L	E	
	E3–34	++	M	I	L	D	L	K	
	E3–38	++	E	P	M	D	L	I	
	E3–48	++	V	M	E	L	Q	G	
	E3–50	++	Y	A	M	E	L	R	
	E3–60	++	S	I	Q	A	L	T	
	E3–64	++	E	P	M	S	L	S	
	E3–66	++	R	P	L	Q	I	V	
	E3–71	++		P	A	N	I	K	G
	E3–77	++	V	P	M	N	M	T	
	E3–83	++	A	K	K	A	R	M	
	E3–89	++	E	P	M	S	L	S	
	E3–98	++		P	M	T	L	A	G
	E3–100	++	I	P	L	P	L	T	
	E3–110	++	K	K	V	R	R	V	
	E3–112	++	R	R	V	R	K	V	
	E3–114	++	G	P	L	G	L	H	
C	L–11	−	Y	G	W	T	L	E	
	L–10	−	V	R	N	N	G	E	
	L–9	−	P	W	E	P	L	C	
	L–8	−	N	P	R	Y	I	L	
	L–7	−	N	V	V	D	H	R	
	L–6	−	V	N	P	F	Y	K	

peptides carrying sequences identical to those in the phage clones were prepared, and their kinetic parameters determined. Shown in Table III are the k_{cat}/K_M values for the good and bad substrate controls (peptides 1 and 2). For reference, we also determined k_{cat}/K_M values for known stromelysin substrates. The substrates chosen were 12 (2,4-dinitrophenyl-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2) (14), and 11 (7-methoxycoumarin-4-yl-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (21)). The values obtained are comparable with those previously determined.

The first test peptides prepared were designed directly from phage clones. The k_{cat}/K_M values for these peptides (3–5) are comparable, and in some cases better, than the values for the good control, indicating that the selection scheme yields high quality substrates. In addition, peptide 6 was prepared based on a consensus sequence of the original 12 hits and was also a good substrate for stromelysin. When an additional 31 positive clones were sequenced, we noted the residues favored in each position (Fig. 6), and made appropriate substitutions in preparing synthetic peptides 7–10. Converting P_4 to Ala from Arg (peptide 7) gave a 5-fold increase in k_{cat}/K_M, whereas replacing Leu with Met in P'_1 (peptide 10) gave only a modest (about 30%) increase in activity. In the P_1 position, Glu dominated over Ala. When we substitute the P_1 Ala with Glu (peptide 9), we saw a 3-fold increase in activity. Interestingly, Niedzwiecki *et al.* (17) found Ala favored of Gln at P_1; they did not test Glu, however. As noted earlier, the P_3 position was dominated by Pro. The only other residue found repeatedly in the B position was Ala, although at much lower frequency. When Ala was substituted for Pro at P_3 (peptide 8), cleavage rates dropped about 3-fold, similar to the result seen previously (17). The results in Table III show that not only can we obtain new peptide substrate sequences from the positive clones, but that the consensus data obtained (Fig. 6*A*) can also yield valuable information in the design of substrates.

Effect of Peptide Length—Although previous studies of MMP substrate optimization generally found that peptides need only extend from P_3 to P'_3 to maintain activity, the peptides designated as optimized substrates were always heptaptides or longer (16, 17). Since our peptides were highly optimized already, we decided to test our consensus substrates as hexapeptides. Peptide 13 is a truncation of peptide 7 (except for the substitution of Leu with Ala, since the Leu was from flanking phage sequence). As can be seen in Table III, this truncation has only a modest effect on activity. By again substituting Glu for Ala in P_1, we see a doubling of activity, yielding the most active stromelysin substrate described to date.[4] This result clearly shows that MMP substrates do not have to be comprised of 7 or more amino acids to have good activity.

Screening the Library with Matrilysin—After the successful screening of the phage library with sfSTR, we wanted to further test the system with a second protease. We chose the MMP matrilysin in order to see if the selection could identify sites where selectivity could be engineered into peptide substrates. The library was subjected to three rounds of screening, and the nucleotide sequences of 23 clones designated as positive by the phage proteolysis assay were determined. The deduced protein sequences are shown in Table IIB, and the corresponding frequency analysis is shown in Fig. 6*B*. Although the patterns are similar to those seen for sfSTR, there are some interesting

[4] Niedzwiecki *et al.* (17) described the octapeptide Arg-Pro-Lys-Pro-Leu-Ala*Phe-TrpNH$_2$ (* = site of cleavage), with a k_{cat}/K_M of 18,000 M^{-1} s^{-1}, which was the best substrate described using only natural amino acids (interestingly, that substrate is very similar in sequence to clone C4–33). The best stromelysin substrate currently reported is *N*-(2,4-dinitrophenyl)-Arg-Pro-Lys-Pro-Leu-Ala*Nva-Trp-NH$_2$ (Nva = norvaline), also from Niedzwiecki *et al.* (17), which has a k_{cat}/K_M of 45,000 M^{-1} s^{-1}.

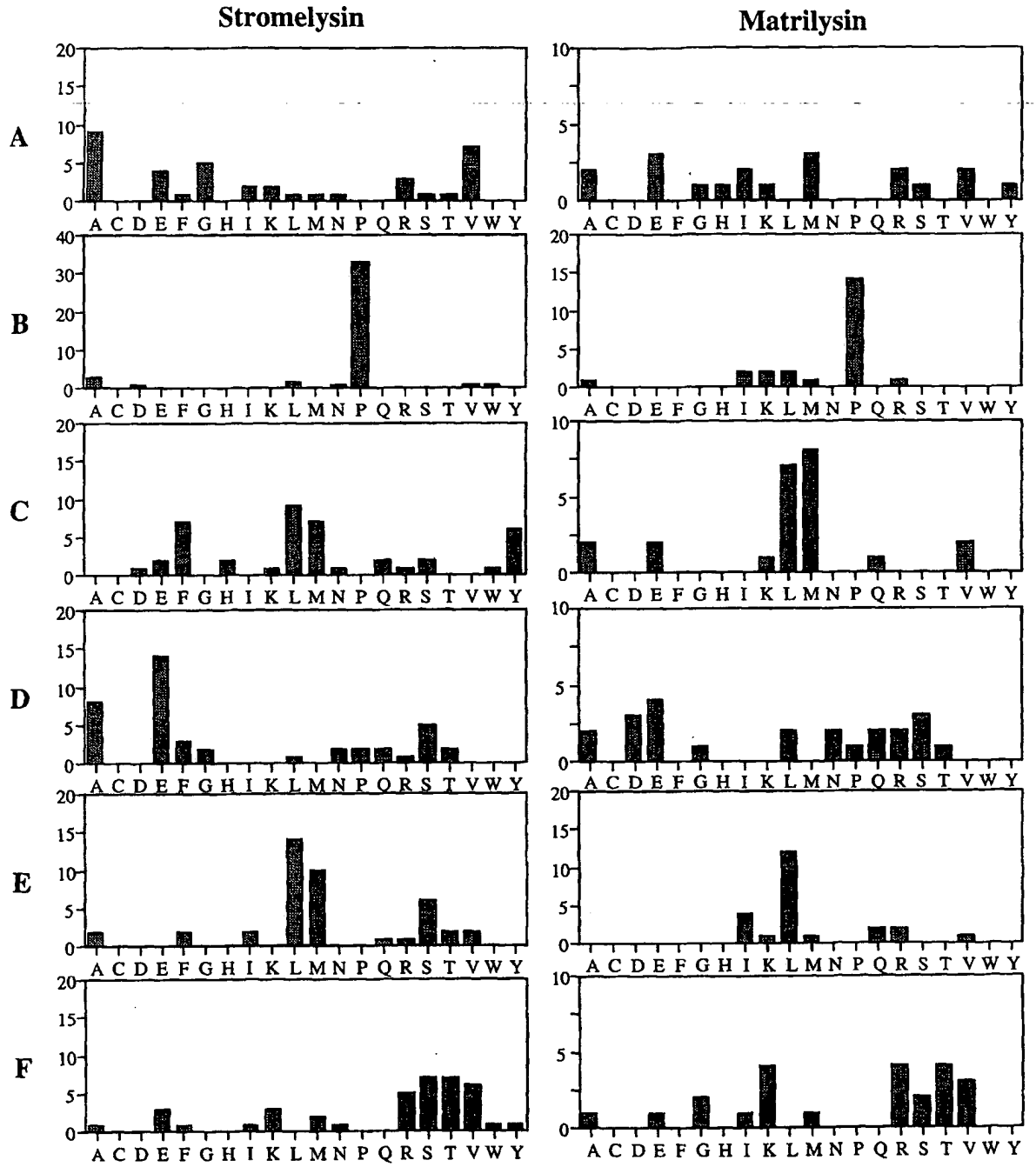

FIG. 6. **Frequency analysis of substrate phage clones for *stromelysin* (*left*) and *matrilysin* (*right*) listed in Table III.** For each position in the N_6 library protease target sequence (*A-F*), the frequency of occurrence for each of the 20 natural amino acids is shown. The *y* axis indicates the number of times a particular residue occurred in that position. Note that the scale for each position ranges from 0 to 20 clones, except for *position B* (under *stromelysin*), which had been reduced for 0–40 clones, and *positions A, C, D,* and *F* (under *matrilysin*), which had been magnified for 0–10 clones.

differences. In particular, at position E, the predominant residues are Leu and Met for sfSTR. In contrast, while Leu remained predominant in that position for matrilysin, only 1 out of 23 clones carried a Met. Likewise, in position E, Leu and Met were predominant for both enzymes, but Phe and Tyr, while frequent for sfSTR, were completely absent in matrilysin. These biases predict that matrilysin will show selectivity *versus* stromelysin at the P_2 and P'_1 positions. These predictions are borne out by the kinetic analysis of the peptides with matrilysin. Comparison of peptides 6 and 10, which differ only in the P'_1 position, shows that the substitution of Met for Leu results in a modest increase in activity for sfSTR. In contrast, this change causes an 8-fold decrease in activity for matrilysin. Analogous results are seen for peptides 14 and 15. The substitution here of Phe for Leu results in a 3-fold decrease in the k_{cat}/K_M value for sfSTR, but a nearly 3-fold increase for matrilysin. Again, peptides 15 and 16 are the smallest and most active peptide substrates of matrilysin described to date.

Quantitative Proteolysis Assay—The phage proteolysis assay described above is useful for assessing qualitatively whether

TABLE III

Kinetic parameters of synthetic peptides based on phage clones

Peptides corresponding to the literature good or bad sequences, various positive clones, or the consensus of the earliest phage clones were synthesized and characterized with sfSTR as described under "Materials and Methods." Peptides 3–10 were prepared in the framework Ac-G-*X*-*X*-*X*-*X*-*X*-*X*-L-V to match the context they were in when carried by the phage. Amino acids that are italicized indicate a variation from the consensus sequence (either 6, 13, or 14) above it. · indicates the site of cleavage for sfSTR as determined by mass spectrometric analysis of the cleavage products. *, not determined. Peptides 11 and 12 are literature standards (see text); the values obtained for them here are comparable with those determined previously (21). Mca, 7-methoxycoumarin-4-yl; DNP, 2,4-dinitrophenyl.

No.	Based on clone	Subsite									Stromelysin			Matrilysin		
		P_5	P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M	K_M	k_{cat}
											$M^{-1}\,s^{-1}$	mM	s^{-1}	$M^{-1}\,s^{-1}$	mM	s^{-1}
1	Good	Ac	Gly	Pro	Leu	Ala ·	Leu	Trp	Ala	Leu	2,700	1.5	4	*	*	*
2	Bad	Ac	Asn	Pro	Val	Glu	Pro	Ala			*	*	*		*	*
3	5–2	Ac	Gly	Ile	Pro	Phe	Glu ·	Gln	Arg	Leu	Val	11,000	1.4	15	425	3.7	1.6
4	3–9	Ac	Gly	Arg	Pro	Gln	Thr	Leu	Ala	Leu	Val	3,200	*	*	4,130	0.40	1.8
5	3–3 and 6–5	Ac	Gly	Arg	Pro	His	Gln	Val	Val	Leu	Val	880	*	*		*	*
6	Consensus	Ac	Gly	Arg	Pro	Phe	Ala ·	Leu	Arg	Leu	Val	15,000	0.40	6	30,600	0.40	13
7	Consensus 2	Ac	Gly	*Ala*	Pro	Phe	Ala	Leu	Arg	Leu	Val	80,000	*	*	61,800	0.20	10
8	Consensus 3	Ac	Gly	Arg	*Ala*	Phe	Ala ·	Leu	Arg	Leu	Val	4,400	*	*	13,300	0.80	10
9	Consensus 4	Ac	Gly	Arg	Pro	Phe	*Glu*	Leu	Arg	Leu	Val	52,000	0.20	10	50,000	0.24	11
10	Consensus 5	Ac	Gly	Arg	Pro	Phe	Ala	*Met*	Arg	Leu	Val	20,000	0.75	16	3,700	1.3	4.7
11	Literature		Mca	Pro	Leu	Gly	Leu	DPA	Ala Arg	$-NH_2$	16,000	*	*	55,000	*	*
12	Literature		DNP	Pro	Leu	Gly	Leu	Trp	Darg	$-NH_2$	2,000	*	*	*	*	*
13	Hexapep 1		Ac-	Pro	Phe	Ala	Leu	Arg	Ala	$-NH_2$	62,000	0.10	8	23,700	0.50	12
14	Hexapep 2		Ac-	Pro	Phe	*Glu*	Leu	Arg	Ala	$-NH_2$	126,000	0.08	10	56,000	0.26	15
15	Hexapep 3		Ac-	Pro	*Leu*	Glu	Leu	Arg	Ala	$-NH_2$	42,000	0.30	13	177,000	0.05	10
16	Hexapep 4		Ac-	Pro	*Met*	Glu	Leu	Arg	Ala	$-NH_2$	38,500	0.45	17	148,000	0.14	20

the peptide sequence carried by a phage is cleaved by the protease. By collecting the dot-blot assay data in a quantitative manner, it should be possible to rank order the various phage substrates. To obtain quantitative data, phage proteolysis assays were stopped and dotted at different time points and the blot scanned by laser densitometry. The relative "phage concentration" (the level of phage retaining the reporter tag) at each time point, $[P]$, was determined by comparison of dot intensity to a serial dilution of untreated phage.[5] By plotting log $[P]$ *versus* time for each of the substrate phage clones, we can obtain a set of slopes corresponding to first-order decay rates. Since we are observing loss of substrate at a constant enzyme concentration, knowledge of the initial substrate concentrations is not required (29). Thus, while we cannot determine absolute k_{cat}/K_M values, the comparison of the decay rates enables us to determine the *relative* k_{cat}/K_M values of the individual clones:

$$\frac{\text{slope}_A}{\text{slope}_B} = \frac{(k_{cat}/K_m)_A}{(k_{cat}/K_m)_B} \qquad \text{(Eq. 1)}$$

The data for three clones, as well as fTC-Good and Bad, is shown in Fig. 7. The decrease of log $[P]$ with time is plotted. The slope for each curve was determined by linear regression and plotted *versus* the k_{cat}/K_M values (Fig.7, *inset*) for the known synthetic peptides (from Table III). The relationship is no t an ideal line, suggesting that the structure of the protein flanking the 6 amino acids in the target region influences relative to the k_{cat}/K_M values for the peptides on the phage. Thus, while the assay may not be able to discriminate between clones with similar k_{cat}/K_M values (fTC-Good, clones A3–3 and A3–9), the data do indicate that the assay is fairly predictive in distinguishing poor (fTC-Bad) from highly active substrates (clone A5–2).

DISCUSSION

The discovery of peptide substrates for proteases has traditionally been a slow and expensive exercise, requiring the synthesis and testing of large numbers of synthetic peptides. Recently, several groups have developed innovative methods for preparing and analyzing large numbers of peptides as protease substrates. A number of these methods utilize a pool of chemically synthesized peptides and are used to screen substitutions in one or two positions at a time (30–33). A few methods use recombinant techniques and offer the opportunity to screen large number of peptides (34–36). None of these techniques offered a practical approach to screening very large numbers of substrates until the efforts of Matthews and Wells (13) made it possible to screen $>10^7$ pentameric substrates at once. We have here presented an analogous system that we have used to screen $>10^8$ hexameric sequences.

In addition, we have introduced a method for assaying putative substrate clones. This assay is simple, rapid, and requires only very small amounts (100 μl) of culture supernatant. With the phage proteolysis assay, the degree of enrichment achieved in each round of selection is easily monitored, allowing adjustment of the selection conditions (*i.e.* protease concentration) for each subsequent round without delaying the selection process. Moreover, the use of solution phase digests allows us to have precise control over enzyme and substrate concentrations, which is not possible in assay systems where one or more of the assay components is immobilized on beads or microtiter plates.

We have tested our system with recombinant forms of the MMPs stromelysin and matrilysin, as we know that substitutions in any of the six positions from P_3 to P'_3 will affect the specificity constant of the peptide substrates. This is the only information we used to start our screen. Our goal was to find substrates as potent as the current literature standards (8, 16–18). We were able to find clones that were as good or better than these standards (clones A5–2 and A3–3, Tables II and III). Due to the large number of sequences obtained, we could identify trends toward certain amino acids in some positions. Using the consensus sequence suggested by the positive phage clones available at the time, we also designed and tested five consensus peptides (6–10). We were pleased to find that these consensus peptides were better than the literature standards, and that after having prepared only four synthetic peptides based on consensus results, we had achieved a k_{cat}/K_M value nearly

[5] We have noted that the reactivity of the phage with the detecting antibody varies from clone to clone. Thus, no attempt was made to determine absolute phage concentrations.

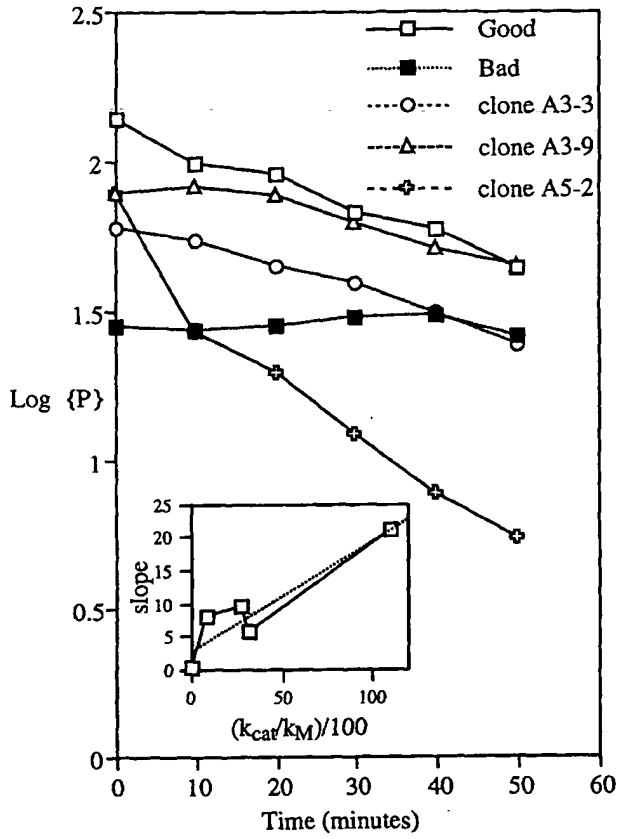

FIG. 7. **Quantitative analysis of the proteolytic analysis of phage clones.** Preparations of phage clones A3–3, A3–9, A5–2, and fTC-Good and Bad were made and titered. Equivalent titers of each clone were treated with sfSTR at 5 μg/ml at 37 °C, and the reaction stopped at the time points indicated, dotted onto nitrocellulose, and detected as in Fig. 5. The blot was then scanned by laser densitometry, and the relative phage concentration at each time point was determined by comparison to a serial 2-fold dilution of untreated phage. The log of {*P*} for each clone is shown at the different time points. *Inset*, the slope of each decay curve is plotted against the known k_{cat}/K_M values of the synthetic peptides (from Table III).

20-fold better than our positive control. In fact, peptides 14 and 15 represent both the smallest *and* the most active peptide substrates of stromelysin and matrilysin, respectively, described to date.

The use of the phage system also gave us an unexpected insight into sites of selectivity between the two enzymes. While initial examination of the data reveals that these proteases have overlapping substrate specificity, closer inspection of the phage and peptide results indicates that certain subsites show distinct preferences: in particular, the opposing preferences for Phe and Leu at P_2 (peptides 14 and 15) and Leu and Met at P'_1 (peptides 6 and 10). Thus, while we learned of the similarity in substrate preferences for the two enzymes, there was sufficient data generated to allow the construction of substrates with differential sensitivities (see for example, peptide 3).

One important piece of information that cannot be derived from the peptides while they are on phage is the location of the site of peptide cleavage. We were only able to determine this once synthetic peptides were prepared and analyzed. It is thus interesting that the sequences obtained "lined up" so well in that the vast majority of the clones had Pro in their B position. Without this invariant residue, lining up the hits to build a consensus sequence might have been more difficult. It is unclear why the predicted P_3 position was nearly always located in B, and only rarely in A. Possibilities include biases due to the sequence flanking the random N_6 region, as well those due to steric hindrance from the tethers and pIII protein.

Another advantage of the phage systems over the screening of pools of synthetic peptides is that we acquire discreet rather than averaged data. The synthetic screening methods described above yield only averaged results for each position, as summarized for the phage data in Fig. 6. The discreet results, shown in Table II, allow us to search for trends which might indicate interactions between one or more subsites. For example, the most abundant amino acids in the D position (P1) were Glu, followed by Ala. Examination of Table II shows that when D = Glu, the residue in the C (P2) position is generally a bulky hydrophobic residue (Tyr, Leu, Met, and Phe). In contrast, when D = Ala, there is no marked preference. Another example can be shown for the eight cases where C = Met, where in six of these seven, E (P'2) is also Met. Yet when C instead is the closely related reside Leu (in nine of the hits), there is no such correlation: the most common residue in the E position in these cases is the hydrophilic Ser (three times) followed by Leu (twice) and then other residues. Although the significance of such correlations remains to be tested, they are intriguing and can lead to models that can be further tested using newly designed substrates. However, these correlations can be made with only with techniques that yield discreet results.

The differences in the approaches used for the use of monovalent and polyvalent phage as protease substrate discovery tools invites comparison of the two systems. While the monovalent system has been shown to be quite useful, the polyvalent system possesses certain advantages: 1) all phage can act as substrate phage in the polyvalent system. In monovalent systems, it is estimated that only 10% of the phage particles carry one copy of the recombinant pIII protein (25). This increases the effective substrate concentration in the polyvalent system by at least 50-fold, thus increasing the sensitivity of the system (due to higher concentration of substrate at a given level of phage). Polyvalent phage preps give stronger signal on Western blots, making the phage proteolysis assay possible. 2) Since 90% of the monovalent phage do not carry pIII fusions, the non-recombinant phage lacking the tether must be removed prior to selection. This is accomplished by immobilizing the recombinant phage in microtiter plates coated with tether-binding protein and treating the phage with protease while immobilized. Polyvalent phage, being 100% recombinant, are digested in solution rather than immobilized on a solid surface. The advantages of this are: (i) there is little restriction on number of phage that can be screened in solution, but the surface system limits the number of phage that can be routinely immobilized on microtiter plates. Scale up of the solution phase system is thus very convenient when significantly larger libraries are prepared (*i.e.* in our first experiment, 10^{11} phage were treated in a single reaction); (ii) protease resistance of tether binding protein (*i.e.* mAb) is not an issue; (iii) solution proteolysis offers more precise control of cleavage conditions. This has proven especially useful in the quantitative dot-blot assay.

The major disadvantage of the polyvalent system described here is the appearance of non-reactive phage clones, which does not occur in the monovalent system because of the pre-binding step which essentially eliminates clones with defective epitopes. Nothing about the polyvalent method, however, precludes our use of a binding step in later rounds to eliminate non-reactive clones.

In summary, we describe a system that can be used for the routine isolation of new substrates for poorly characterized endoproteases. The system is simple and rapid. Few assump-

tions about the nature of the selected protease need be made (what is the true physiological substrate; is it a serine or cysteine protease, etc). In fact, the protease need not be pure; it should only be free of other proteolytic (or protease inhibiting) activities. Filamentous phage are valuable tools for studying proteases as they are generally protease resistant. We have found no nonspecific degradation due to stromelysin, matrilysin, HIV protease, and tissue type plasminogen activator.[6] If a protease is found that degrades the phage, one should be able to treat the phage vector with an excess of protease and select for mutant phage that have become resistant to proteolysis and use this modified vector to prepare a new substrate library.

Acknowledgments—We would like to thank Lois Aldwin and Grace Hsu for providing us with synthetic peptides for these studies. mAb 179 was a gift of Ron Barrett. We would like to thank Bob Gray for peptide 11 as well as much helpful advice, Bill Fitch for the mass spectrometry analysis of the peptide products, Zhengyu Yuan for advice, and Bill Dower for valuable comments on the manuscript.

Note Added in Proof—A highly active peptide substrate for stromelysin with a k_{cat}/K_M value of 218,000 was recently identified (Nagase, H., Fields, C. G., and Fields, G. B. (1994) *J. Biol Chem.* **269,** 20952–20957). Although this substrate is more active than our best substrate, it does contain 11 amino acids (some of which are unnatural) and is thus much larger than our substrates.

REFERENCES

1. Fersht, A. R. (1985) *Enzyme Structure and Mechanism,* pp. 105–106, Freeman, New York
2. Fields, G. B., Netzel-Arnett, S. J., Windsor, L. J., Engler, J. A., Birkedal-Hansen, H., and Van Wart, H. E. (1990) *Biochemistry* **29,** 6670–6677
3. Hellen, C. U., Krausslich, H. G., and Wimmer, E. (1989) *Biochemistry* **28,** 9881–9890
4. Krausslich, H. G., Ingraham, R. H., Skoog, M. T., Wimmer, E., Pallai, P. V., and Carter, C. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 807–811
5. Netzel-Arnett, S., Fields, G., Birkedal-Hansen, H., and Van Wart, H. E. (1991) *J. Biol. Chem.* **266,** 6747–6755
6. Griffiths, J. T., Phylip, L. H., Konvalinka, J., Strop, P., Gustchina, A., Wlodawer, A., Davenport, R. J., Briggs, R., Dunn, B. M., and Kay, J. (1992) *Biochemistry* **31,** 5193–5200
7. Tözsér, J., Weber, I. T., Gustchina, A., Bláha, I., Copeland, T. D., Louis, J. M., and Oroszlan, S. (1992) *Biochemistry* **31,** 4793–4800
8. Teahan, J., Harrison, R., Izquierdo, M., and Stein, R. L. (1989) *Biochemistry* **28,** 8497–8501
9. Poorman, R. A., Tomasselli, A. G., Heinrikson, R. L., and Kezdy, F. J. (1991) *J. Biol. Chem.* **266,** 14554–14561
10. Tomasselli, A. G., Hui, J. O., Adams, L., Chosay, J., Lowery, D., Greenberg, B., Yem, A., Deibel, M. R., Zurcher-Neely, H., and Heinrikson, R. L. (1991) *J. Biol. Chem.* **266,** 14548–14553
11. Hoess, R. H. (1993) *Curr. Opin. Struct. Biol.* **3,** 572–579
12. Scott, J. K. (1992) *Trends Biochem. Sci.* **17,** 241–245
13. Matthews, D. J., and Wells, J. A. (1993) *Science* **260,** 1113–1117
14. Stack, M. S., and Gray, R. D. (1989) *J. Biol. Chem.* **264,** 4277–4281
15. Seltzer, J. L., Akers, K. T., Weingarten, H., Grant, G. A., McCourt, D. W., and Eisen, A. Z. (1990) *J. Biol. Chem.* **265,** 20409–20413
16. Netzel-Arnett, S., Sang, Q. X., Moore, W. G. I., Navre, M., Birkedalhansen, H., and Van Wart, H. E. (1993) *Biochemistry* **32,** 6427–6432
17. Niedzwiecki, L., Teahan, J., Harrison, R. K., and Stein, R. L. (1992) *Biochemistry* **31,** 12618–12623
18. Netzel-Arnett, S., Mallya, S. K., Nagase, H., Birkedal-Hansen, H., and Van Wart, H. E. (1991) *Anal. Biochem.* **195,** 86–92
19. Cwirla, S. E., Peters, E. A., Barrett, R. W., and Dower, W. J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6378–6382
20. Gramsch, C., Meo, T., Riethmüller, G., and Herz, A. (1983) *J. Neurochemistry* **40,** 1220–1226
21. Knight, C. G., Willenbrock, F., and Murphy, G. (1992) *FEBS Lett.* **296,** 263–266
22. Parmley, S. F., and Smith, G. P. (1988) *Gene (Amst.)* **73,** 305–318
23. Scott, J. K., and Smith, G. P. (1990) *Science* **249,** 386–390
24. Devlin, J. J., Panganiban, L. C., and Devlin, P. E. (1990) *Science* **249,** 404–406
25. Bass, R., Greene, R., and Wells, J. A. (1990) *Proteins* **8,** 309–314
26. Model, P., and Russel, M. (1988) in *The Bacteriophages* (Calendar, R., ed) pp. 375–456, Plenum Press, New York
27. Lowman, H. B., Bass, S. H., Simpson, N., and Wells, J. A. (1991) *Biochemistry* **30,** 10832–10838
28. Barrett, R. W., Cwirla, S. E., Ackerman, M. S., Olson, A. M., Peters, E. A., and Dower, W. J. (1992) *Anal. Biochem.* **204,** 357–364
29. Schellenberger, V., Siegel, R. A., and Rutter, W. J. (1993) *Biochemistry* **32,** 4344–4348
30. Petithory, J. R., Masiarz, F. R., Kirsch, J. F., Santi, D. V., and Malcolm, B. A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 11510–11514
31. Birkett, A. J., Soler, D. F., Wolz, R. L., Bond, J. S., Wiseman, J., Berman, J., and Harris, R. B. (1991) *Anal. Biochem.* **196,** 137–143
32. Berman, J., Green, M., Sugg, E., Anderegg, R., Millington, D. S., Norwood, D. L., McGeehan, J., and Wiseman, J. (1992) *J. Biol. Chem.* **267,** 1434–1437
33. Schellenberger, V., Turck, C. W., Hedstrom, L., and Rutter, W. J. (1993) *Biochemistry* **32,** 4349–4353
34. Smith, T. A., and Kohorn, B. D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5159–5162
35. Baum, E. Z., Bebernitz, G. A., and Gluzman, Y. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 10023–10027
36. Dasmahapatra, B., Didomenico, B., Dwyer, S., Ma, J., Sadowski, I., and Schwartz, J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 4159–4162
37. Marcy, A. I., Eiberger, L. L., Harrison, R., Chan, H. K., Hutchinson, N. I., Hagmann, W. K., Cameron, P. M., Boulton, D. A., and Hermes, J. D. (1991) *Biochemistry* **30,** 6476–6483

[6] L. Ding, M. Smith, and M. Navre, unpublished results.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 47, Issue of November 21, pp. 29449–29453, 1997
Printed in U.S.A.

Specific Proteolysis of the Kinase Protein Kinase C-related Kinase 2 by Caspase-3 during Apoptosis

IDENTIFICATION BY A NOVEL, SMALL POOL EXPRESSION CLONING STRATEGY*

(Received for publication, September 5, 1997)

Vincent L. Cryns‡§, Youngsup Byun‡, Ajay Rana¶, Harry Mellor‖, Kevin D. Lustig‡, Louis Ghanem¶, Peter J. Parker‖, Marc W. Kirschner‡, and Junying Yuan‡**

From the ‡Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, the ¶Diabetes Unit, Massachusetts General Hospital, Charlestown, Massachusetts 02129, and the ‖Protein Phosphorylation Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, United Kingdom

The caspase family of proteases plays a critical role in the execution of apoptosis. However, efforts to decipher the molecular mechanisms by which caspases induce cell death have been greatly hindered by the lack of systematic and broadly applicable strategies to identify their substrates. Here we describe a novel expression cloning strategy to rapidly isolate cDNAs encoding caspase substrates that are cleaved during apoptosis. Small cDNA pools (approximately 100 clones each) are transcribed/translated *in vitro* in the presence of [^{35}S]methionine; these labeled protein pools are then incubated with cytosolic extracts from control and apoptotic cells. cDNA pools encoding proteins that are specifically cleaved by the apoptotic extract and whose cleavage is prevented by the caspase inhibitor acetyl-Tyr-Val-Ala-Asp chloromethylketone are subdivided and retested until a single cDNA is isolated. Using this approach, we isolated a partial cDNA encoding protein kinase C-related kinase 2 (PRK2), a serine-threonine kinase, and demonstrate that full-length human PRK2 is proteolyzed by caspase-3 at Asp^{117} and Asp^{700} *in vitro*. In addition, PRK2 is cleaved rapidly during Fas- and staurosporine-induced apoptosis *in vivo* by caspase-3 or a closely related caspase. Both of the major apoptotic cleavage sites of PRK2 *in vivo* lie within its regulatory domain, suggesting that its activity may be deregulated by proteolysis.

Caspases are a novel family of cysteine proteases with aspartate specificity that are related to the *Caenorhabditis elegans* cell death gene product CED-3. Evidence from many laboratories indicates that caspases play a critical role in the execution of apoptosis. Ectopic expression of these proteases induces programmed cell death. Caspases are normally present in cells as catalytically inactive proenzymes and are proteolytically processed and activated during the induction of apoptosis. Moreover, viral, peptide, and dominant negative inhibitors of caspases delay or prevent programmed cell death (reviewed in Ref. 1). Finally, homozygous inactivation of caspase-1 (2) and caspase-3 (3) in mice results in selective defects in apoptosis.

Because caspase activation is a crucial event in apoptosis, it is essential to identify the downstream molecular targets of these proteases whose selective proteolysis is likely to underlie the characteristic morphological features of apoptotic cell death. Although a number of structural and signaling proteins have been shown to be cleaved by caspases during programmed cell death (reviewed in Ref. 4), our understanding of the molecular mechanisms by which caspases induce cell death has been greatly hindered by the lack of systematic and broadly applicable strategies to identify these substrates. Given the absence of such methods and the growing number of caspase family members, it seems likely that the majority of apoptotic caspase targets have yet to be identified.

In this report, we describe a method to identify caspase substrates directly and rapidly using labeled protein pools derived from small cDNA library pools (5) that have been transcribed/translated *in vitro* (6). These protein pools are incubated with cell-free extracts prepared from nonapoptotic and apoptotic cells. cDNA pools encoding proteins specifically cleaved by an apoptotic extract (and prevented by caspase inhibitors) are subdivided and re-examined until a single cDNA is isolated. Using this approach, we demonstrate that the serine-threonine kinase PRK2[1] (7) is rapidly and specifically cleaved by caspase-3 during the induction of apoptotic cell death.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Human Jurkat cells were grown in RPMI 1640 medium (Life Technologies, Inc.) with 10% heat-inactivated fetal calf serum (FCS). Acetyl-Tyr-Val-Ala-Asp chloromethyl ketone (YVAD-CMK), acetyl-Tyr-Val-Ala-Asp aldehyde (YVAD-CHO), and acetyl-Asp-Glu-Val-Asp aldehyde (DEVD-CHO) were obtained from BACHEM Bioscience, Inc. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma. Stock solutions were prepared according to the manufacturer's instructions.

Preparation of ^{35}S-Labeled Protein Pools—Pre-existing cDNA pools of approximately 100 clones each from a *Xenopus* gastrula-stage library (5) were transcribed and translated *in vitro* using the TnT Coupled

* This work was supported in part by Mentored Clinical Scientist Development Award K08-CA01752-04 (to V. L. C.) and Grants GM26875 (to M. W. K.) and AG12859-01 (to J. Y.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF027183.

§ Present address: Div. of Endocrinology, Metabolism and Molecular Medicine, Northwestern University Medical School, Tarry 15-755, 303 E. Chicago Ave., Chicago, IL 60611.

** To whom correspondence should be addressed: Dept. of Cell Biology, LHRRB 409, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115. Tel.: 617-432-4170; Fax: 617-432-4177; E-mail: jyuan@warren.med.harvard.edu.

[1] The abbreviations used are: PRK2, protein kinase C-related kinase 2; FCS, fetal calf serum; YVAD-CMK, acetyl-Tyr-Val-Ala-Asp chloromethylketone; YVAD-CHO, acetyl-Tyr-Val-Ala-Asp aldehyde, DEVD-CHO, acetyl-Asp-Glu-Val-Asp aldehyde; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PAGE, polyacrylamide gel electropheresis; mAb, monoclonal antibody.

Reticulocyte Lysate System (Promega) in the presence of [^{35}S]methionine and SP6 RNA polymerase. Reactions were scaled down from the manufacturer's instructions to a 5-μl volume using 100–500 ng of total DNA/pool; all other reagents were proportionately reduced.

Preparation of Control and Apoptotic Cytosolic Extracts—Jurkat cells were grown to near confluence in RPMI 1640 with 10% FCS and concentrated to 2×10^7 cells/ml in fresh medium immediately prior to making extracts. An aliquot of concentrated cells was treated with 250 ng/ml of anti-Fas monoclonal antibody (mAb) CH-11 (Kamiya Biomedical Company, Thousand Oaks, CA) for 1 h. Cytosolic extracts from untreated cells (control extract) and anti-Fas-treated cells (apoptotic extract) were prepared as described previously (6) and stored in aliquots at −80 °C until used. Each μl of extract (control or apoptotic) was derived from 4×10^5 cells and contained approximately 8–10 μg of total protein.

Proteolytic Cleavage Reactions—Protein pools were analyzed in triplicate. 1.5-μl aliquots of each ^{35}S-labeled protein pool were incubated for 1 h at 37 °C with 5 μl of (i) control extract, (ii) apoptotic extract, or (iii) apoptotic extract preincubated for 15 min with 10 μM YVAD-CMK, a specific caspase inhibitor that inhibits many caspases under these conditions (8). Cleavage reactions were stopped by the addition of an equal volume of 2 × protein lysis buffer (125 mM Tris-Cl (pH 6.8), 2% SDS, 20% glycerol and 10% β-mercaptoethanol) and boiled for 5 min. The protein products were separated by SDS-PAGE and visualized by autoradiography as detailed previously (6). Each time cleavage reactions were run, ^{35}S-labeled poly(ADP-ribose) polymerase (PARP) (prepared from pBSK-PARP using the TnT Coupled Reticulocyte Lysate System), a well characterized caspase-3 substrate (9, 10), was incubated with the above-noted three cleavage conditions to verify the specificity of the cleavage activity (*i.e.*, present in the apoptotic extract only) and its sensitivity to inhibition by the caspase inhibitor YVAD-CMK.

Identification of Putative Apoptotic Caspase Substrates—Labeled protein pools containing putative caspase substrates cleaved during apoptosis were identified by (i) the disappearance of a protein in the apoptotic extract that was present in both the control extract and in the apoptotic extract preincubated with caspase inhibitor and/or (ii) the appearance of a novel protein fragment in the apoptotic extract only. Once a positive protein pool was confirmed, the corresponding cDNA pool was progressively subdivided and re-examined in the same manner until a single positive cDNA clone was isolated (6). Positive clones were sequenced and examined for homology to known sequences by internet BLAST search (National Center for Biotechnology Information).

Proteolytic Cleavage of Human PRK2 in Vitro—pcDNA3 plasmids containing full-length human PRK2 and PRK1 cDNAs were transcribed and translated *in vitro* in the presence of [^{35}S]methionine using the TnT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. ^{35}S-Labeled PRK2 and PRK1 were incubated with 10 μl of control and apoptotic extracts and analyzed as above. ^{35}S-Labeled PRK2 was also incubated for 1 h at 37 °C with apoptotic extracts that had been preincubated for 15 min with either DEVD-CHO (1–100 nM) or YVAD-CHO (0.1–10 μM). In addition, ^{35}S-labeled PRK2 was incubated with bacterially expressed caspase-1, caspase-2, and caspase-3, and the cleavage products were analyzed as described previously (8, 11); a catalytically inactive mutant caspase-2 containing a serine substitution of the active site cysteine residue was used as a control in these studies (11).

Determination of Caspase-3 Cleavage Sites in PRK2 in Vitro—Based on the size of the observed cleavage fragments and examination of the amino acid sequence of human PRK2 for potential caspase-3 cleavage sites (DXXD motifs) (12, 13), two mutant human PRK2 constructs, D117A and D700E, were made using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions with the following oligonucleotide primers: 5′-GAAGATATTACAGCATGCCCAAGGACTCC-3′ and 5′-GGAGTCCTTGGGCATGCTGTAATATCTTC-3′ (D117A) 5′-GAGATGAAGTAGAAAGCCTGATGTGTG-3 and 5′-CACACATCAGGCTTTCTACTTCATTCTC-3′ (D700E). All constructs were verified by automated DNA sequencing of both strands. Mutant PRK2 cDNA constructs were transcribed and translated *in vitro*, and the corresponding ^{35}S-labeled proteins were incubated with extracts and bacterially expressed caspases as above.

Induction of Apoptosis and Determination of Cell Viability—Jurkat cells were seeded overnight in 96-well plates at a density of 5×10^4 cells/well in phenol red-free RPMI 1640 with 10% FCS. Cells were treated with 250 ng/ml anti-Fas mAb CH-11 for 0, 1, 2, 4, 6, or 8 h or 2 μM staurosporine (Sigma) for 0, 1, 2, 4, or 6 h. For caspase inhibitor studies, Jurkat cells were pretreated for 2 h with either DEVD-CHO (1–100 μM) or YVAD-CHO (1–100 μM) and then treated for an additional 5 h with 250 ng/ml anti-Fas mAb. Viabilities were determined in quadruplicate in two independent experiments by the MTT conversion assay as described previously (8).

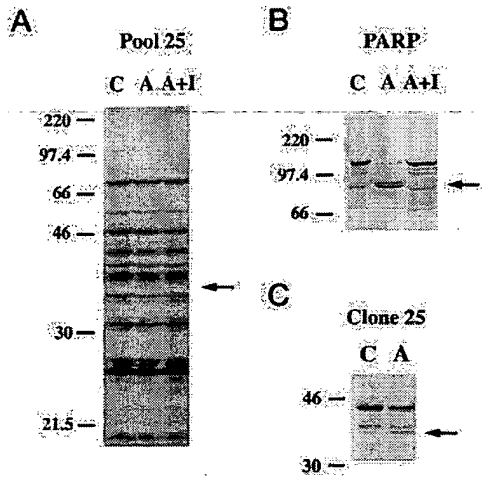

FIG. 1. **Isolation of PRK2 as a putative, apoptotic caspase substrate by small pool expression cloning.** *A*, appearance of a novel protein fragment of approximately 36 kDa (indicated by an *arrow*) following incubation of ^{35}S-labeled protein pool 25 with an apoptotic extract (*lane A*) for 1 h at 37 °C that was not observed after incubation of this same pool with control extract (*lane C*) or apoptotic extract preincubated with 10 μM YVAD-CMK (*lane A+I*). *B*, specific cleavage of ^{35}S-labeled PARP into its characteristic 85-kDa fragment (indicated by an *arrow*) by the apoptotic extract (*lane A*) but not by the apoptotic extract preincubated with 10 μM YVAD-CMK (*lane A+I*). *C*, isolation of the single cDNA from pool 25 whose ^{35}S-labeled protein product is cleaved into the above-noted 36-kDa fragment (indicated by an *arrow*) by the apoptotic extract (*lane A*). cDNA pool 25 was progressively subdivided, and the corresponding labeled protein pools were re-examined as above until a single cDNA was identified. Sequencing of this cDNA revealed that it is a *Xenopus* homologue of PRK2 (7). Preparation of cytosolic extracts and ^{35}S-labeled proteins, the cleavage reaction conditions, SDS-PAGE separation of proteins, and protein visualization by autoradiography are detailed under "Experimental Procedures." The molecular mass of markers in kDa is indicated at the *left* of each panel.

Western Blotting—Cell lysates were prepared and analyzed by Western blotting as described elsewhere (8). The following antibodies were used in these studies: (i) a PRK2 polyclonal peptide antibody recognizing the C terminus of human PRK2 (1:200 dilution) (7); (ii) PARP mAb C2–10 (Pharmingen) (1:2000 dilution); (iii) caspase-3 (CPP32) mAb (Transduction Laboratories) (1:1000 dilution); and (iv) α-tubulin mAb (Sigma) (1:10,000 dilution).

RESULTS AND DISCUSSION

75 cDNA pools containing approximately 100 cDNAs each were transcribed/translated *in vitro* in the presence of [^{35}S]methionine, usually resulting in the production of only 15–28 labeled proteins/pool. This discrepancy likely reflects the presence of empty or noncoding inserts in the cDNA library and variable transcription/translation efficiencies among cDNAs, some of which are not full-length and lack transcription initiation sequences (6). The labeled protein pools were then examined for protein bands specifically modified by a cell-free apoptotic extract derived from cells treated with anti-Fas monoclonal antibody. These cytosolic extracts have been shown to faithfully reproduce many of the characteristic features of apoptosis observed *in vivo*, including selective proteolysis of caspase substrates and fragmentation of added nuclei (14–16). Of the 75 labeled protein pools screened, five contained a single protein that was cleaved by the apoptotic extract but not by this same extract preincubated with the caspase inhibitor YVAD-CMK; one pool contained two such proteins (data not shown). In this report, we describe the isolation and characterization of one of these proteins.

As shown in Fig. 1*A*, incubation of ^{35}S-labeled protein pool 25 with an apoptotic extract (*lane A*) resulted in the production of

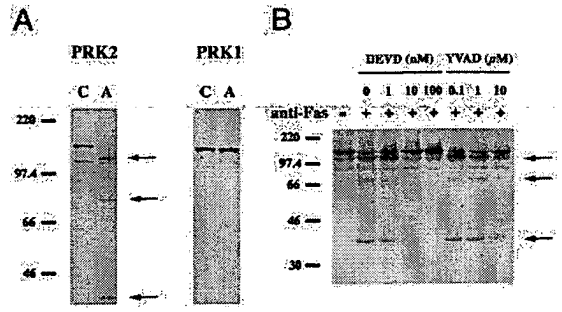

FIG. 2. **Specific proteolysis of human PRK2 in apoptotic extracts by a caspase(s) sensitive to inhibition by DEVD-CHO.** *A*, cleavage of PRK2 but not the closely related kinase PRK1 by an apoptotic extract (*lane A*). ^{35}S-Labeled PRK2 and PRK1 were prepared by coupled *in vitro* transcription/translation from pcDNA3 plasmids containing full-length human PRK2 and PRK1 cDNAs, respectively, and incubated with 10 μl of control (*lane C*) or apoptotic (*lane A*) extract for 1 h at 37 °C. *B*, cleavage of PRK2 by an apoptotic extract is inhibited by DEVD-CHO. ^{35}S-Labeled PRK2 was incubated as above with control extracts or apoptotic extracts preincubated for 15 min with 0–100 nM DEVD-CHO or 0.1–10 μM YVAD-CHO. Labeled proteins were separated by SDS-PAGE and visualized by autoradiography as described under "Experimental Procedures." The molecular mass of markers in kDa is indicated at the *left* of each panel, and the cleavage fragments are indicated by *arrows* at the *right* of each panel.

a novel protein fragment of approximately 36 kDa (indicated by the arrow) that was not observed when this pool was incubated with either control extract (*lane C*) or apoptotic extract preincubated with the caspase inhibitor YVAD-CMK (*lane A+I*). The specificity of the proteolytic activity of the apoptotic extract (*lane A*) and its sensitivity to inhibition by YVAD-CMK (*lane A+I*) was verified by using ^{35}S-labeled PARP, a well characterized caspase-3 substrate, as a control (Fig. 1*B*). The observed pattern for protein pool 25 suggests that the novel protein band seen in the apoptotic extract represents a cleavage fragment of a caspase substrate. In this case, disappearance of the intact caspase substrate in the apoptotic extract was not observed because it was only partially cleaved (Fig. 1*C*). cDNA pool 25 was progressively subdivided into smaller pools, and the smaller pools were retested until a single cDNA encoding a protein with the above-noted cleavage pattern was isolated (Fig. 1*C*). DNA sequencing of clone 25 revealed that it is a partial *Xenopus* PRK2 cDNA containing the entire C-terminal kinase domain. Comparison of clone 25 with human PRK2 (7) revealed a striking degree of homology in the kinase domains: >90% identity at the amino acid level.

Because clone 25 is a partial *Xenopus* PRK2 cDNA, we next examined whether full-length human PRK2 was cleaved by the apoptotic extract. As shown in Fig. 2*A* (*left-hand panel*), ^{35}S-labeled human PRK2 was completely cleaved by the apoptotic extract (*lane A*) into three fragments of approximately 110, 70, and 36 kDa (indicated by the *arrows*). However, the closely related kinase PRK1 was not cleaved by the apoptotic extract (Fig. 2*A*, *right-hand panel*). Moreover, the apoptotic cleavage of PRK2 *in vitro* was completely inhibited by 10 nM DEVD-CHO (Fig. 2*B*), a peptide caspase inhibitor that preferentially inhibits caspase-3 and related subfamily members (10). In contrast, the apoptotic proteolysis of PRK2 was only partially prevented by 1000-fold higher (10 μM) concentrations of YVAD-CHO (Fig. 2*B*), a selective inhibitor of caspase-1 and related subfamily members (17). Taken together, these results indicate that human PRK2 is cleaved at two distinct sites by a DEVD-inhibitable caspase (caspase-3 or a caspase-3-like family member) during apoptosis *in vitro*.

In an effort to better characterize the caspase(s) responsible for PRK2 proteolysis in the apoptotic extract, we incubated ^{35}S-labeled human PRK2 with caspase-1, caspase-2, and

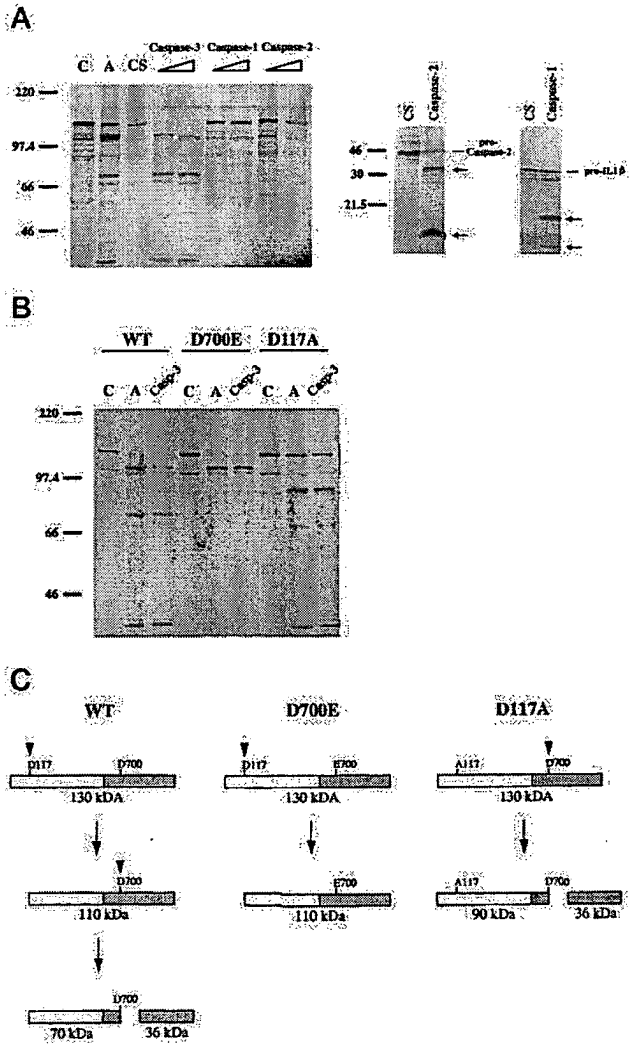

FIG. 3. **Human PRK2 is cleaved *in vitro* by caspase-3 at Asp^{117} and Asp^{700}.** *A*, specific proteolysis of PRK2 by caspase-3 into fragments of the same size as those observed in the apoptotic extract. In the *left-hand panel*, ^{35}S-labeled PRK2 was incubated for 1 h at 37 °C with control extract (*lane C*), apoptotic extract (*lane A*), bacterial lysate (179.2 μg of total protein) containing catalytically inactive caspase-2 (*lane CS*), caspase-3 lysate (33.7 or 168.4 μg of total protein), caspase-1 lysate (35.3 or 176.4 μg of total protein), or caspase-2 lysate (36.9 or 184.6 μg of total protein). In the *right-hand panel*, the activity of caspase-2 and caspase-1 was verified by incubating ^{35}S-labeled procaspase-2 with 36.9 μg of caspase-2 lysate and ^{35}S-labeled pro-interleukin-1β with 35.3 μg of caspase-1 lysate for 1 h at 37 °C. The cleavage fragments are indicated by *arrows*. *B*, PRK2 mutants D700E and D117A are resistant to cleavage by caspase-3 at these sites, respectively. PRK2 mutant constructs D700E and D117A were created by site-directed mutagenesis as detailed under "Experimental Procedures." ^{35}S-Labeled wild-type PRK2 (*WT*), D700E and D117A were incubated as above with control extract (*lane C*), apoptotic extract (*lane A*), or 33.7 μg caspase-3 lysate (*Casp-3*). The cleavage products were separated by SDS-PAGE and detected by autoradiography as described under "Experimental Procedures." The molecular mass of markers in kDa is indicated at the *left* of each panel. *C*, schematic representation of caspase-3-mediated cleavage of wild type, D700E, and D117A PRK2 proteins *in vitro*. The N-terminal regulatory domain of PRK2 is shown as a *white bar*, whereas the C-terminal kinase domain is *shaded*. Cleavage sites are indicated by *arrowheads*, and the approximate size of the resulting fragments is shown. Of note, the putative N-terminal fragment (<20 kDa) generated by cleavage at Asp^{117} was not visualized (see *panel B*) and is not drawn.

caspase-3 (the prototypical members of the three caspase subfamilies). Bacterially expressed caspase-3 cleaved PRK2 into three fragments of identical size seen in the apoptotic extracts

(Fig. 3*A*, *left-hand panel*). However, incubation of PRK2 with amounts of caspase-1 or caspase-2 that were 5-fold greater than that needed to cleave pro-interleukin-1β and procaspase-2, respectively, did not result in any detectable proteolysis of PRK2 (Fig. 3*A*, *both panels*). These findings are consistent with the inhibitor profile of the PRK2 protease and strongly implicate caspase-3 as the protease responsible for PRK2 cleavage during apoptosis *in vitro*.

To identify the caspase-3 cleavage sites in human PRK2, we examined its amino acid sequence and identified two DXXD motifs (the consensus caspase-3 cleavage sequence (12, 13)) that would result in cleavage fragments of the expected size: DITD^{117}C (in the N-terminal regulatory domain) and DEVD^{700}S (in the C-terminal kinase domain). By site-directed mutagenesis of the critical aspartic acid residues, we created two mutant PRK2 constructs (D117A and D700E). As shown in Fig. 3*B*, substitution of aspartate 700 with glutamic acid (D700E) prevented cleavage at this site (the 70- and 36-kDa cleavage fragments were not formed) but not at the second site (the 110-kDa cleavage fragment was produced). In contrast, substitution of aspartate 117 with alanine (D117A) prevented cleavage at this site (the 110-kDa fragment was not formed) but did not prevent cleavage at Asp^{700}, thereby resulting in cleavage fragments of 36 kDa and approximately 90 kDa (appropriately larger than the corresponding 70-kDa fragment seen in wild-type PRK2 because the 20-kDa N-terminal cleavage site is missing). These cleavage patterns are illustrated schematically in Fig. 3*C*. Interestingly, cleavage of full-length PRK2 in the D117A mutant was reproducibly diminished compared with either wild-type PRK2 or the D700E mutant (Fig. 3*B*), suggesting that proteolysis at this N-terminal site may facilitate subsequent cleavage at Asp^{700}. These results unambiguously establish Asp^{117} and Asp^{700} as the two sites in human PRK2 cleaved by caspase-3 during apoptosis *in vitro*.

Having demonstrated that human PRK2 is cleaved by caspase-3 *in vitro*, we next wanted to determine whether PRK2 was cleaved during apoptosis *in vivo*. To this end, we performed Western analysis of PRK2 in lysates from Jurkat cells treated with anti-Fas monoclonal antibody or staurosporine for various time periods. Using a polyclonal peptide antibody directed against the C terminus of human PRK2 (7), a 110-kDa cleavage fragment (indicated by an *arrow*) was readily detected within 2 h of treatment with either anti-Fas mAb or staurosporine (Fig. 4*A*) and was observed as early as 1 h after these treatments on prolonged exposure of the blot (data not shown). This fragment is identical in size to the N-terminal cleavage fragment produced *in vitro* by cleavage at Asp^{117} (data not shown). A smaller fragment of approximately 100 kDa (indicated by an *arrow*) was first observed 4 h after treatment with anti-Fas mAb and 2 h after staurosporine treatment. In addition, several faint, smaller fragments (approximately 50–70 kDa in size) were observed at later time points (after 4 h), especially in staurosporine-treated cells; these minor fragments are likely produced by proteases activated after the initiation of apoptosis. Of note, no cleavage fragment in the 30–40-kDa range was detected during Fas- or staurosporine-induced cell death *in vivo*, indicating that proteolysis at Asp^{700} does not occur during apoptosis *in vivo*. Within 6 h of anti-Fas treatment or 4 h of staurosporine treatment, virtually all of the full-length PRK2 had been proteolyzed into the above-noted cleavage fragments.

Importantly, PRK2 proteolysis occurs early during the induction of Fas- and staurosporine-mediated apoptosis. As noted, the 110-kDa cleavage fragment can be detected within 1–2 h of exposure to either of these apoptotic stimuli. Within this time period, 88–96% of the anti-Fas mAb-treated cells and 52–75% of the staurosporine-treated cells are still viable. Furthermore,

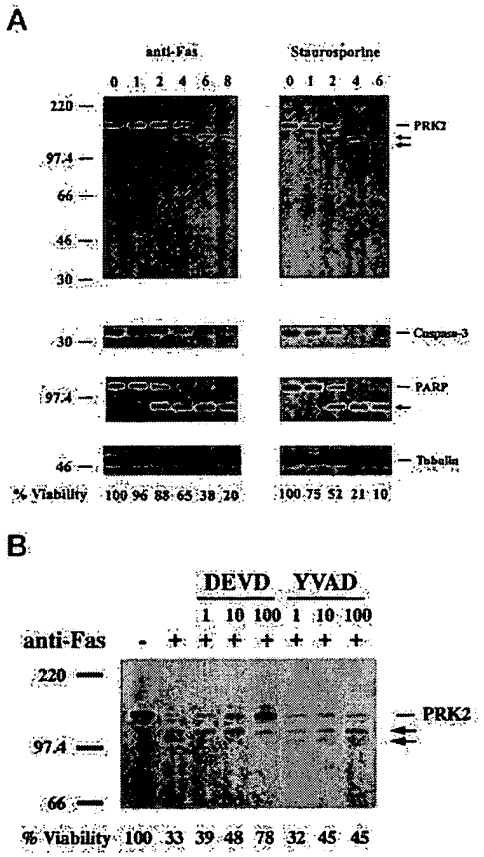

FIG. 4. **Specific and rapid cleavage of PRK2 during Fas- and staurosporine-induced apoptosis *in vivo* by a caspase(s) sensitive to inhibition by DEVD-CHO.** *A*, similar time course for proteolytic cleavage of PRK2, caspase-3, and PARP during Fas- and staurosporine-induced apoptosis *in vivo*. Jurkat cells were treated with 250 ng/ml anti-Fas mAb or 2 μM staurosporine for 0–8 h or 0–6 h, respectively. Cell lysates were prepared and subsequently analyzed by Western blotting using the appropriate antibodies as detailed under "Experimental Procedures." α-Tubulin was used as a control to verify that equivalent amounts of protein were loaded in each lane. *B*, Fas-induced proteolysis of PRK2 is potently inhibited by DEVD-CHO. Jurkat cells were preincubated for 2 h with 0–100 μM DEVD-CHO or YVAD-CHO and then treated for an additional 5 h with 250 ng/ml anti-Fas mAb. For each condition, the corresponding cell viability was determined by MTT conversion assay (8) and is indicated at the *bottom*. The molecular mass of markers in kDa is indicated at the *left* of each panel.

at all time points examined, the extent of PRK2 proteolysis correlated with the degree of cell death. Fas- and staurosporine-induced PRK2 cleavage also coincided temporally with caspase-3 proteolytic activation (as manifested by reduction in intensity of its proenzyme) and proteolysis of the well characterized caspase-3 substrate PARP (Fig. 4*A*), suggesting that caspase-3 (or a related subfamily member) is the protease responsible for PRK2 cleavage during apoptosis *in vivo*. Further evidence implicating caspase-3 in this role comes from the *in vivo* inhibitor studies. Preincubation of cells with 100 μM DEVD-CHO, a peptide inhibitor that preferentially inhibits caspase-3 (10), prevented Fas-induced PRK2 cleavage and cell death (Fig. 4*B*). The same concentration of YVAD-CHO, a caspase-1 selective inhibitor (17), had little effect on Fas-mediated PRK2 cleavage and cell death. Interestingly, the proteolytic formation of the smaller, 100-kDa PRK2 cleavage fragment was particularly sensitive to inhibition by DEVD-CHO. Concentrations of DEVD-CHO as little as 1–10 μM inhibited formation of this fragment and resulted in the accumulation of excess amounts of the larger, 110-kDa cleavage fragment (Fig.

4*B*), suggesting that apoptotic PRK2 proteolysis *in vivo* may be executed by two distinct caspase-3-like proteases with different sensitivities to DEVD-CHO.

In short, the serine-threonine kinase PRK2 is rapidly and specifically cleaved by caspase-3 (and/or a caspase-3-like subfamily member) during apoptosis *in vitro* and *in vivo*. Although one of the cleavage sites of PRK2 *in vitro* lies within its kinase domain (D700), both of its major apoptotic cleavage sites *in vivo* lie within its N-terminal regulatory domain. Given the evidence that both PRK2 and the closely related kinase PRK1 are activated by limited tryptic proteolysis (18, 19), presumably by removal of an inhibitory N-terminal domain (20), it is tempting to speculate that the activity of PRK2 might be deregulated by its cleavage during apoptosis. Indeed, several other kinases, including protein kinase C δ (21, 22) and θ (23), the p21-activated kinase PAK2 (24), and MEKK-1 (25), are cleaved and activated by caspase-3 during apoptotic cell death; proteolytic activation of these kinases may contribute to the morphological manifestations of apoptosis.

In addition, the successful isolation of PRK2 as a caspase substrate cleaved during apoptosis demonstrates the utility of the small pool expression cloning strategy presented here. By providing a rapid and direct method to identify the downstream targets of these critical apoptotic proteases, this approach should enable investigators to systematically dissect the molecular events/signaling pathways that play an important role in the execution of apoptosis. Although we used an apoptotic extract to screen for caspase substrates, individual recombinant caspases could be used to identify substrates of a particular caspase. Furthermore, the approach has much broader potential applications and can be used to identify substrates of a variety of enzymatic activities, including kinases (6, 26). We are currently exploring several of these applications in our studies of apoptotic signal transduction.

Acknowledgments—We are indebted to Drs. H. Li, L. Bergeron, T. McGarry, R. King, and T. Stukenberg for advice and to Drs. S. Vincent and J. Settleman for providing plasmids.

REFERENCES

1. Fraser, A., and Evan, G. (1996) *Cell* **85,** 781–784
2. Kuida, K., Lippke, J. A., Ku, G., Harding, M. W., Livingston, D. J., Su, M. S.-S., and Flavell, R. A. (1995) *Science* **267,** 2000–2002
3. Kuida, K., Zheng, T. S., Na, S., Kuan, C.-Y., Yang, D., Karasuyama, H., Rakic, P., and Flavell, R. A. (1996) *Nature* **384,** 368–372
4. Rosen, A., and Casciola-Rosen, L. (1997) *J. Cell. Biochem.* **64,** 50–54
5. Lustig, K., Kroll, K., Sun, E., and Kirschner, M. (1996) *Development* **122,** 4001–4012
6. Lustig, K. D., Stukenberg, T., McGarry, T., King, R. W., Cryns, V. L., Mead, P., Zon, L., Yuan, J., and Kirschner, M. W. (1997) *Methods Enzymol.* **283,** 83–99
7. Palmer, R. H., Ridden, J., and Parker, P. J. (1995) *Eur. J. Biochem.* **227,** 344–351
8. Cryns, V. L., Bergeron, L., Zhu, H., Li, H., and Yuan, J. (1996) *J. Biol. Chem.* **271,** 31277–31282
9. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994) *Nature* **371,** 346–347
10. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T.-T., Yu, V. L., and Miller, D. K. (1995) *Nature* **376,** 37–43
11. Li, H., Bergeron, L., Cryns, V., Pasternack, M., Zhu, H., Shi, L., Greenberg, A., and Yuan, J. (1997) *J. Biol. Chem.* **272,** 21010–21017
12. Talanian, R. V., Quinlan, C., Trautz, S., Hackett, M. C., Mankovich, J. A., Banach, D., Ghayur, T., Brady, K. D., and Wong, W. W. (1997) *J. Biol. Chem.* **272,** 9677–9682
13. Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T., and Nicholson, D. W. (1997) *J. Biol. Chem.* **272,** 17907–17911
14. Lazebnik, Y. A., Cole, S., Cooke, C. A., Nelson, W. G., and Earnshaw, W. C. (1993) *J. Cell Biol.* **123,** 7–22
15. Martin, S. J., Newmeyer, D. D., Mathias, S., Farschon, D. M., Wang, H. G., Reed, J. C., Kolesnick, R. N., and Green, D. R. (1995) *EMBO J.* **14,** 5191–5200
16. Enari, M., Hase, A., and Nagata, S. (1995) *EMBO J.* **14,** 5201–5208
17. Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R., Aunins, J., Elliston, K. O., Ayala, J. M., Casano, F. J., Chin, J., Ding, G. J.-F., Egger, L. A., Gaffney, E. P., Limjuco, G., Palyha, O. C., Raju, S. M., Rolando, A. M., Salley, J. P., Yamin, T.-T., Lee, T. D., Shively, J. E., MacCross, M., Mumford, R. A., Schmidt, J. A., and Tocci, M. J. (1992) *Nature* **356,** 768–774
18. Yu, W., Liu, J., Morrice, N. A., and Wettenhall, R. E. H. (1997) *J. Biol. Chem.* **272,** 10030–10034
19. Mukai, H., Kitagawa, M., Shibata, H., Takanaga, H., Mori, K., Shimakawa, M., Miyahara, M., Hirao, K., and Ono, Y. (1994) *Biochem. Biophys. Res. Commun.* **204,** 348–356
20. Kitagawa, M., Shibata, H., Toshimori, M., Mukai, H., and Ono, Y. (1996) *Biochem. Biophys. Res. Commun.* **220,** 963–968
21. Emoto, Y., Manome, Y., Meinhardt, G., Kisaki, H., Kharbanda, S., Robertson, M., Ghayur, T., Wong, W. W., Kamen, R., Weichselbaum, R., and Kufe, D. (1995) *EMBO J.* **14,** 6148–6156
22. Ghayur, T., Hugunin, M., Talanian, R. V., Ratnofsky, S., Quinlan, C., Emoto, Y., Pandey, P., Datta, R., Huang, Y., Kharbanda, S., Allen, H., Kamen, R., Wong, W., and Kufe, D. (1996) *J. Exp. Med.* **184,** 2399–2404
23. Datta, R., Kojima, H., Yoshida, K., and Kufe, D. (1997) *J. Biol. Chem.* **272,** 20317–20320
24. Rudel, T., and Bokoch, G. (1997) *Science* **276,** 1571–1574
25. Cardone, M. H., Salvesen, G. S., Widmann, C., Johnson, G., and Frisch, S. M. (1997) *Cell* **90,** 315–323
26. Stukenberg, P., Lustig, K., McGarry, T., King, R., Kuang, J., and Kirschner, M. (1997) *Curr. Biol.* **7,** 338–348

LEXSEE 314 F3D 1313

AMGEN INC., Plaintiff-Cross Appellant, v. HOECHST MARION ROUSSEL, INC. (now known as Aventis Pharmaceuticals Inc.) and TRANSKARYOTIC THERAPIES, INC., Defendants-Appellants.

01-1191, 01-1218

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

314 F.3d 1313; 2003 U.S. App. LEXIS 118; 65 U.S.P.Q.2D (BNA) 1385

January 6, 2003, Decided

SUBSEQUENT HISTORY: Rehearing denied by, Rehearing, en banc, denied by *Amgen Inc. v. Hoechst Marion Roussel, Inc., 2003 U.S. App. LEXIS 5401* (Fed. Cir., Mar. 3, 2003)
On remand at, Motion granted by, Motion denied by *Amgen, Inc. v. Hoechst Marion Roussel, Inc., 287 F. Supp. 2d 126, 2003 U.S. Dist. LEXIS 19953* (D. Mass., 2003)
On remand at, Patent interpreted by *Amgen, Inc. v. Hoechst Marion Roussel, Inc., 2004 U.S. Dist. LEXIS 22943* (D. Mass., Oct. 15, 2004)

PRIOR HISTORY: [**1] Appealed from: The United States District Court for the District of Massachusetts. Chief Judge William G. Young. *Amgen, Inc. v. Hoechst Marion Roussel, Inc., 126 F. Supp. 2d 69, 2001 U.S. Dist. LEXIS 431* (D. Mass., 2001)

DISPOSITION: AFFIRMED IN PART, VACATED IN PART, REMANDED.

CASE SUMMARY:

PROCEDURAL POSTURE: In this patent infringement action, defendant, the alleged patent infringer, appealed and plaintiff, the patent holder, cross-appealed from a judgment of the United States District Court for the District of Massachusetts, which construed the disputed claims; held each of the patents enforceable; held two patents valid and infringed; and held two patents not infringed.

OVERVIEW: The holder owned numerous patents directed to the production of erythropoietin, a naturally occurring hormone that controlled the formation of red blood cells in bone marrow. The patent holder marketed and sold a highly successful commercial embodiment of the patented erythropoietin. The holder sought to impede the alleged infringers from commercializing a competitive erythropoietin product. The alleged infringer argued that the patents in suit were all unenforceable, that the district court's claim construction was erroneous, and alternatively, if that claim construction was correct, that the court's validity determinations were erroneous. The instant court held that the district court's finding that the patent holder satisfied the written description requirement was not clearly erroneous. The court reasoned that the district court properly weighed the testimony and found that the evidence showed that the descriptions adequately described to those of ordinary skill in the art in 1984 the use of the broad class of available mammalian and vertebrate cells to produce the claimed high levels of human erythropoietin in culture.

OUTCOME: The court affirmed in toto the district court's claim construction. The court also affirmed the district court's determination that none of the patents in suit were unenforceable for inequitable conduct. However, the court found that the district court misapplied the law, and vacated the judgment in several respects. The decision was also remanded for reconsideration of several issues.

LexisNexis(R) Headnotes

Patent Law > Infringement Actions > Claim Interpretation > General Overview

[HN1] Because patent claim language defines claim scope, the first step in an infringement analysis is to construe the claims, i.e., to determine the scope and meaning of that which is allegedly infringed. To properly construe the claims, a court must examine the claims, the rest of the specification, and, if in evidence, the prosecution history. Thereafter, the properly construed claims are compared to the accused product or process to determine whether each of the claim limitations is met, either literally or equivalently.

Patent Law > Jurisdiction & Review > Standards of Review > General Overview
[HN2] An appellate court considers the trial court's claim construction -- a matter of law -- afresh on appellate review.

Patent Law > Claims & Specifications > Claim Language > General Overview
[HN3] It is the patent claims that measure the invention. Because the claims are best understood in light of the specification of which they are a part, however, courts must take extreme care when ascertaining the proper scope of the claims, lest they simultaneously import into the claims limitations that were unintended by the patentee. The danger of improperly importing a limitation is even greater when the purported limitation is based upon a term not appearing in the claim. If courts once begin to include elements not mentioned in the claim in order to limit such claim, courts would never know where to stop.

Patent Law > Claims & Specifications > Claim Language > General Overview
Patent Law > Infringement Actions > Claim Interpretation > General Overview
[HN4] When a patent claim does not contain a certain limitation and another claim does, that limitation cannot be read into the former claim in determining either validity or infringement. There is a rebuttable presumption that different claims are of different scope.

Patent Law > Claims & Specifications > Claim Language > General Overview
Patent Law > Infringement Actions > Claim Interpretation > General Overview
[HN5] Courts indulge a heavy presumption that a claim term carries its ordinary and customary meaning. Although the prosecution history is always relevant to claim construction, it is also true that the prosecution history may not be used to infer the intentional narrowing of a claim absent the applicant's clear disavowal of claim coverage, such as an amendment to overcome a rejection.

Patent Law > Infringement Actions > Claim Interpretation > General Overview
Patent Law > Claims & Specifications > Claim Language > General Overview
[HN6] Patent claims are not perforce limited to the embodiments disclosed in the specification.

Patent Law > Claims & Specifications > Claim Language > General Overview
[HN7] Patentees can use negative limitations such as "non-human" and "non-natural" to avoid rejection under *35 U.S.C.S. § 101.*

Patent Law > Infringement Actions > General Overview
Patent Law > Claims & Specifications > Description Requirement > General Overview
Patent Law > U.S. Patent & Trademark Office Proceedings > Reissues > General Overview
[HN8] It is axiomatic that patent claims are construed the same way for both invalidity and infringement. But because the features of the accused product or process are often undisputed, this axiom invites a common approach in the appellate arguments by accused infringers: the principal argument challenges the correctness of a trial court's broad claim construction; the contingent argument, assuming the trial court's claim construction is affirmed, challenges validity under *35 U.S.C.S. § 112,* para. 1 of the asserted patents in light of that broad construction.

Patent Law > Claims & Specifications > Best Mode > Adequate Disclosure
Patent Law > Claims & Specifications > Enablement Requirement > Standards & Tests
Patent Law > Claims & Specifications > Description Requirement > General Overview
[HN9] *35 U.S.C.S. § 112* describes what must be contained in the patent specification. Among other things, it must contain a written description of the invention, and of the manner and process of making and using it such as to enable any person of ordinary skill in the art to which it pertains to make and use the same. *35 U.S.C.S. § 112,* para. 1. Thus, this statutory language mandates satisfaction of two separate and independent requirements: an applicant must both describe the claimed invention adequately and enable its reproduction and use. Third, he must disclose what he considers the best mode of practicing his invention.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Claims & Specifications > Definiteness > General Overview

Patent Law > Claims & Specifications > Description Requirement > General Overview

[HN10] The purpose of the written description requirement is to prevent an applicant from later asserting that he invented that which he did not; the applicant for a patent is therefore required to recount his invention in such detail that his future claims can be determined to be encompassed within his original creation. Satisfaction of this requirement is measured by the understanding of the ordinarily skilled artisan. Compliance with the written description requirement is essentially a fact-based inquiry that will necessarily vary depending on the nature of the invention claimed. Because of its fact intensive nature, an appellate court reviews a district court's decision on the adequacy of written description for clear error.

Patent Law > Claims & Specifications > Claim Language > Combination Claims
Patent Law > U.S. Patent & Trademark Office Proceedings > Filing Requirements > Drawings
Patent Law > Claims & Specifications > Description Requirement > General Overview

[HN11] A broadly drafted patent claim must be fully supported by the written description and drawings.

Patent Law > Claims & Specifications > Description Requirement > General Overview

[HN12] A patentee need only describe the invention as claimed, and need not describe an unclaimed method of making the claimed product.

Patent Law > Claims & Specifications > Enablement Requirement > Proof
Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
Patent Law > Jurisdiction & Review > Standards of Review > General Overview

[HN13] The enablement requirement is often more indulgent than the written description requirement. The specification need not explicitly teach those in the art to make and use the invention; the requirement is satisfied if, given what they already know, the specification teaches those in the art enough that they can make and use the invention without "undue experimentation." Enablement is a question of law; an appellate court therefore reviews the trial court's determination de novo, deferring to its assessment of subsidiary facts underlying the legal question unless clearly erroneous.

Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
Patent Law > Claims & Specifications > Enablement Requirement > General Overview

[HN14] Where the method is immaterial to the patent claim, the enablement inquiry simply does not require the specification to describe technological developments concerning the method by which a patented composition is made that may arise after the patent application is filed. Thus, the specification's failure to disclose the later-developed endogenous activation technology cannot invalidate the patent.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Claims & Specifications > Claim Language > General Overview

[HN15] The law makes clear that the specification need teach only one mode of making and using a claimed composition.

Patent Law > Jurisdiction & Review > Standards of Review > General Overview

[HN16] Appellate courts are largely limited on review to deciding whether those findings based on that testimony are clearly erroneous. Appellate courts may, of course, review de novo the district court's interpretation of precedent.

Patent Law > Infringement Actions > Summary Judgment > Appeals
Patent Law > Infringement Actions > Claim Interpretation > General Overview
Patent Law > Jurisdiction & Review > Subject Matter Jurisdiction > Appeals

[HN17] The second step of the infringement analysis is comparison of the properly construed claims to the accused product or process. A court's review of this step differs depending upon whether the issue of infringement was resolved on summary judgment or after a full trial. In the case of summary judgment, the court reviews de novo the trial court's finding that there was no genuine issue as to any material fact regarding infringement. *Fed. R. Civ. P. 56(c)*. After a full bench trial, infringement is a question of fact that a court reviews, of course, for clear error. When JMOL is entered under *Fed. R. Civ. P. 52(c)*, the appellate court reviews the district court's determination for clear error, as if it had been entered at the close of all the evidence.

Patent Law > Claims & Specifications > Theory of Invention
Patent Law > Claims & Specifications > Definiteness > Precision Standards
Patent Law > Originality > Joint & Sole Inventorship

[HN18] Under *35 U.S.C.S. § 112*, para. 2, a patent applicant is required, at the close of his specification, to particularly point out and distinctly claim the subject matter the applicant regards as his invention. The

requirement of claim definiteness set out in § 112, para. 2 assures that claims in a patent are sufficiently precise to permit a potential competitor to determine whether or not he is infringing. The standard of indefiniteness is somewhat high; a claim is not indefinite merely because its scope is not ascertainable from the face of the claims. Rather, a claim is indefinite under § 112, para. 2 if it is insolubly ambiguous, and no narrowing construction can properly be adopted.

Patent Law > Claims & Specifications > Definiteness > General Overview

[HN19] A patent claim is indefinite if, when read in light of the specification, it does not reasonably apprise those skilled in the art of the scope of the invention.

Patent Law > Claims & Specifications > Claim Language > General Overview

[HN20] "Comprising" is a term of art used in patent claim language which means that the named elements are essential, but other elements may be added and still form a construct within the scope of the claim. The word "include" means the same thing.

Patent Law > Double Patenting > Elements
Patent Law > Infringement Actions > Defenses > Estoppel & Laches > General Overview

[HN21] A narrowing amendment to satisfy any requirement of the Patent Act may give rise to an estoppel.

Patent Law > Double Patenting > Elements
Patent Law > Infringement Actions > General Overview

[HN22] "Same invention" double patenting is based upon *35 U.S.C.S. § 101*, which states that an inventor may obtain "a patent" for an invention.

Patent Law > U.S. Patent & Trademark Office Proceedings > General Overview
Patent Law > Infringement Actions > Claim Interpretation > General Overview

[HN23] A patent claim interpretation that reads out a preferred embodiment is rarely, if ever, correct and would require highly persuasive evidentiary support.

Patent Law > Infringement Actions > Reverse Doctrine of Equivalents
Patent Law > Infringement Actions > Doctrine of Equivalents > General Overview

[HN24] Under the reverse doctrine of equivalents, an accused product or process that falls within the literal words of a claim nevertheless may not infringe if the product or process is so far changed in principle from a patented article that it performs the same or a similar function in a substantially different way. This doctrine is equitably applied based upon underlying questions of fact, when the accused infringer proves that, despite the asserted claims literally reading on the accused device, it has been so changed that it is no longer the same invention.

Patent Law > Anticipation & Novelty > Description in Patents
Patent Law > Anticipation & Novelty > Description in Publications
Patent Law > Anticipation & Novelty > Elements

[HN25] One of the statutory requirements for patentability is that the invention for which a patent is sought was not known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention by the applicant. *35 U.S.C.S. § 102*(a). Similarly, one is not entitled to a patent if the subject matter of the invention as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which the invention is directed. *35 U.S.C.S. § 103.*

Patent Law > Claims & Specifications > Claim Language > General Overview
Patent Law > Infringement Actions > Claim Interpretation > General Overview

[HN26] A district court may -- indeed, often must -- interpret or define a term in the claims that is not in dispute in order to provide a proper context for the discussion of the terms that are in dispute.

Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
Patent Law > Claims & Specifications > Definiteness > General Overview
Patent Law > Claims & Specifications > Enablement Requirement > General Overview

[HN27] In the context of patent construction, the relevant question is not whether one of ordinary skill would so understand the term, but whether that term should be limited based upon the express disclosure in the specification.

Patent Law > Anticipation & Novelty > General Overview

[HN28] A claimed invention cannot be anticipated by a prior art reference if the allegedly anticipatory disclosures cited as prior art are not enabled.

Patent Law > Anticipation & Novelty > Elements
Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
Patent Law > U.S. Patent & Trademark Office Proceedings > General Overview

[HN29] In patent prosecution the examiner is entitled to reject application claims as anticipated by a prior art patent without conducting an inquiry into whether or not that patent is enabled or whether or not it is the claimed material (as opposed to the unclaimed disclosures) in that patent that are at issue. The applicant, however, can then overcome that rejection by proving that the relevant disclosures of the prior art patent are not enabled.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview

[HN30] Under *35 U.S.C.S. § 103,* a reference need not be enabled; it qualifies as a prior art, regardless, for whatever is disclosed therein.

Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > Elements

[HN31] A patent applicant commits inequitable conduct when, during prosecution of the application, he makes an affirmative representation of a material fact, fails to disclose material information, or submits false material information, and does so with the intent to deceive. As a general principle, materiality and intent are balanced -- a lesser quantum of evidence of intent is necessary when the omission or misrepresentation is highly material, and vice versa. At the same time, however, there must be some threshold showing of intent to be balanced; courts will not find inequitable conduct on an evidentiary record that is completely devoid of evidence of the patentee's intent to deceive the U.S. Patent and Trademark Office.

COUNSEL: Lloyd R. Day, Jr., Day Casebeer Madrid & Batchelder LLP, of Cupertino, California, argued for plaintiff-cross appellant. Of counsel on the brief were Edward M. O'Toole, Howrey Simon Arnold & White, of Chicago, Illinois; Stuart L. Watt, Amgen Inc., of Thousand Oaks, California; and D. Dennis Allegretti, Duane, Morris & Heckscher LLP, of Boston, Massachusetts. Of counsel were Wendy A. Whiteford, Steven M. Odre, Monique L. Cordray, Robert R. Cook, Amgen Inc., of Thousand Oaks, California. Of counsel were David M. Madrid, Robert M. Galvin, Terry L. Tang, Paul S. Grewal, Richard C. Lin, Jonathan Loeb, Jackie N. Nakamura, and Matthew E. Hocker, Day Casebeer Madrid & Batchelder LLP, of Cupertino, California; and Richard M. Wong, Duane Morris & Heckscher LLP, of Boston, Massachusetts.

Herbert F. Schwartz, Fish & Neave, of New York, New York, argued for defendants-appellants. With him on the brief were Kenneth B. Herman, James F. Haley, Jr., Denise L. Loring, Douglas J. Gilbert, Frances M. Lynch, Gerald J. Flattmann, Jr., and [**2] Robert B. Wilson. Of counsel on the brief were Robert S. Frank, Jr. and Eric J. Marandett, Choate, Hall & Stewart, of Boston, Massachusetts. Also of counsel on the brief were Michael J. Astrue and Mary S. Consalvi, Transkaryotic Therapies, Inc., of Cambridge, Massachusetts.

JUDGES: Before MICHEL, CLEVENGER, and SCHALL, Circuit Judges. Opinion for the court filed by Circuit Judge MICHEL. Dissenting opinion filed by Circuit Judge CLEVENGER.

OPINIONBY: MICHEL

OPINION: [*1319] MICHEL, Circuit Judge.

Plaintiff-Cross Appellant Amgen Inc. ("Amgen") is the owner of numerous patents directed to the production of erythropoietin ("EPO"), a naturally occurring hormone that controls the formation of red blood cells in bone marrow. Amgen markets and sells EPOGEN (R), a highly successful commercial embodiment of the patented erythropoietin. Seeking to impede defendants-appellants Hoechst Marion Roussel, Inc. and Transkaryotic Therapies, Inc. (collectively "TKT") from commercializing a competitive EPO product, Amgen filed a declaratory judgment action in the United States District Court for the District of Massachusetts in April 1997, alleging that TKT's Investigational New Drug Application ("INDA") infringed [**3] United States Patent Nos. *5,547,933* ("the *'933* patent"); *5,618,698* ("the *'698* patent"); and *5,621,080* ("the *'080* patent"). The complaint was amended in October 1999 to include United States Patent Nos. *5,756,349* ("the *'349* patent") and *5,955,422* ("the *'422* patent"), which issued after suit was filed.

[*1320] After a three-day Markman hearing, the case was tried to the court for 23 days over the course of four months. In January 2001, the district court issued an exhaustive 244-page opinion in which it: (i) construed the disputed claims; (ii) held each of the patents enforceable; (iii) held the *'080*, *'349* (product claims), and *'422* patents valid and infringed; (iv) held the *'698* patent not infringed; and (v) held the *'933* patent not infringed or, in the alternative, invalid for failure to satisfy *35 U.S.C. § 112. Amgen, Inc. v. Hoescht Marion Roussel, Inc., 126 F. Supp. 2d 69, 57 USPQ2d 1449 (D. Mass. 2001).* On appeal, TKT urges reversal on the grounds that the patents in suit are all unenforceable, that the district court's claim construction was erroneous, and alternatively, if that claim construction was correct, that the court's validity determinations [**4] were erroneous. Amgen asserts, in its cross appeal, that the district court committed error: (i) by comparing the accused process to the examples in the specification rather than the limitations of the method claims of the *'349* and *'698* patents; and (ii) by holding the *'933* patent invalid for

failure to comply with § 112. We heard oral argument on May 7, 2002.

We commend the district court for its thorough, careful, and precise work on what is indubitably a legally difficult and technologically complex case. There is no doubt that the court marshaled tremendous time and resources in its effort to reach correct results. Nevertheless, because we must conclude that the court committed certain errors of law in certain of its validity and infringement determinations, we cannot affirm the judgment in its entirety.

We affirm in toto the district court's claim construction. We also affirm: (i) its determination that none of the patents in suit is unenforceable for inequitable conduct; (ii) its contingent determination that the *'933* patent is invalid under § *112 P 1;* (iii) its grant of summary judgment of infringement of *'422* patent claim 1; (iv) its determination that [**5] the *'080*, *'933*, *'349*, and *'698* patents are not anticipated by the Sugimoto reference; and (v) its determination that *'349* patent claims 1, 3-4, and 6 are infringed. Because the district court misapplied the law, however, we vacate: (i) its determination that the *'933* patent is not infringed; (ii) its determination that the *'080* patent is infringed under the doctrine of equivalents; (iii) its determination that the *'080*, *'349*, and *'422* patents are not invalid; and (iv) its determination that the asserted method claims of the *'698* patent and *'349* patent claim 7 are not infringed. Accordingly, we remand for the district court to reconsider: (i) whether the *'080*, *'349*, and *'422* patents are obvious in light of the Sugimoto prior art or anticipated or obvious in light of the Goldwasser prior art; (ii) whether the *'422* patent is anticipated by Sugimoto reference (and whether Amgen can prove its nonenablement); (iii) whether the asserted claims of the *'698* patent and *'349* patent claim 7 are infringed by the accused method; and (iii) whether the *'080* patent is infringed under the doctrine of equivalents. In sum, as further explained in detail below, we affirm in part, vacate in part, and remand [**6] for further proceedings consistent herewith.

BACKGROUND

As the district court set out in painstaking detail the basics of the underlying technology, we will provide only a brief summary here. The reader's familiarity with the fundamentals of molecular biology, genetics, and recombinant DNA technology necessary to this appeal is presumed. n1

n1 For further reading on these subjects, see generally Robert A. Meyers, ed., Molecular Biology and Biotechnology: A Comprehensive Desk Reference, VCH Publishers (1995); Benjamin Lewin, Genes VII, Oxford Univ. Press (2000); James D. Watson et al., Recombinant DNA (2d ed. 1992).

[*1321] EPO is a naturally occurring protein that initiates and controls erythropoiesis, the production of red blood cells in bone marrow. Red blood cells are critical because they contain hemoglobin, a protein responsible for transporting oxygen from the lungs to peripheral tissues. Because EPO is produced in the kidney, patients with chronic kidney (renal) failure lack normal levels of [**7] EPO and, as a result, have a sub-optimal number of red blood cells -- a condition called anemia. The therapeutic goal for treating anemic patients is to increase the "hematocrit level," which represents the ratio of red blood cells to total blood volume, to normal or near-normal levels. This is accomplished through the introduction of additional EPO into the patient's system.

The implementation of this seemingly simple solution, introduction of exogenous EPO, proved to be difficult. Because human EPO is produced in very small amounts (even from the healthy human kidney), it is difficult to obtain by conventional methods. Early attempts to recover EPO from plasma or from human urine ("urinary EPO" or "uEPO") were unsuccessful because such recovery employed techniques that were complicated, yet still resulted in a low-yield, high-impurity, or unstable EPO end product. *'933* patent, col. 6, line 60 -- col. 7, line 42. Similar attempts using antibody techniques failed because of difficulty in providing for the large-scale isolation of quantities of EPO from mammalian sources sufficient for further analysis, clinical testing, or therapeutic use. Id., col. 9, lines 2-8. The first successful [**8] method of production of a therapeutically effective amount of erythropoietin used recombinant EPO ("rEPO") techniques; Amgen is recognized as the pioneer. See, e.g., Molecular Biology and Biotechnology at 108.

Amgen scientist Dr. Fu-Kuen Lin is the named inventor on all five patents in suit. Instead of attempting to purify EPO from natural sources, Lin isolated and characterized monkey and human EPO genes, then used conventional recombinant DNA technology to produce large amounts of rEPO. *'933* patent, col. 13, lines 50-53. Lin was able to determine the entire DNA sequence of human EPO and from that, its predicted amino acid sequence. Id., Fig. 6; col. 10, lines 65 -- col. 11, line 2. Using the isolated human EPO gene, Lin described several methods for producing therapeutically effective amounts of human EPO using an expression vector. n2 Id., col. 21, line 42 -- col. 25, line 27.

n2 An "expression vector" is a circular piece of DNA (or "plasmid") that is inserted into a host cell to produce (or "express") a protein. The expression vector carries the gene encoding for the protein of interest (in this case human EPO), a marker that assures that the vector is properly introduced into the host cell, and a promoter site that the host will recognize to transcribe the vector's DNA. See generally Thomas E. Crieghton, ed., Encyclopedia of Molecular Biology, vol. 2, John Wiley & Sons, Inc. (1999) at 883-86.

[**9]

EPOGEN (R), the commercial embodiment of Amgen's patented EPO product, is produced by the method disclosed in patent specification Example 10. That example describes the production of human EPO through transfection (introduction) of exogenous DNA into host Chinese hamster ovary ("CHO") cells. The CHO host cell, using its own transcription machinery, then expresses human rEPO in abundance, which then accumulates in the host cell cytoplasm or in the culture media. Id., col. 37, lines 43-49. The rEPO so recovered has the same or similar amino acid sequences and biological properties as naturally [*1322] occurring human EPO, but differs in its "glycosylation," i.e., in the patterns of branched carbohydrate chains that attach to the protein. *'933* patent, col. 10, lines 34-41.

The patents in suit, which all claim priority to a December 1983 application long since abandoned, are continuations of a common ancestor -- United States Patent No. *4,703,008* -- which was at issue in this court's landmark decision in *Amgen Inc. v. Chugai Pharm. Co., 927 F.2d 1200, 18 USPQ2d 1016 (Fed. Cir. 1991).* n3 The *'933* patent issued on August 20, 1996, containing 14 claims drawn primarily [**10] to a non-naturally occurring EPO product with certain characteristics. At issue in this lawsuit are claims 1, 2, and 9 (with the disputed claim terms here and below underscored):

> 1. A non-naturally occurring erythropoietin glycoprotein product having the in vivo biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and having glycosylation which differs from that of human urinary erythropoietin.
>
> 2. The non-naturally occurring EPO glycoprotein product according to claim 1 wherein said product has a higher molecular weight than human urinary EPO as measured by SDS-PAGE.
>
> 9. A pharmaceutical composition comprising an effective amount of a glycoprotein product effective for erythropoietin therapy according to claim 1, 2, 3, 4, 5, or 6 and a pharmaceutically acceptable diluent, adjuvant or carrier.

The *'698* patent issued on April 8, 1997, containing nine claims drawn to a process for producing a glycosylated erythropoietin polypeptide. At issue are claims 4-9:

> 4. A process for the production of a glycosylated erythropoietin polypeptide having the in vivo biological property of causing [**11] bone marrow cells to increase production of reticulocytes and red blood cells comprising the steps:
>
> > a) growing, under suitable nutrient conditions, vertebrate cells comprising promoter DNA, other than human erythropoietin promoter DNA, operatively linked to DNA encoding the mature erythropoietin amino acid sequence of FIG. 6; and
> >
> > b) isolating said glycosylated erythropoietin polypeptide expressed by said cells
>
> 5. The process of claim 4 wherein said promoter DNA is viral promoter DNA.
>
> 6. A process for the production of a glycosylated erythropoietin polypeptide having the in vivo biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells comprising the steps of:
>
> > a) growing, under suitable nutrient conditions, vertebrate cells comprising amplified DNA encoding the mature erythropoietin amino acid sequence of FIG. 6; and

b) isolating said glycosylated erythropoietin polypeptide expressed by said cells.

7. The process of claim 6 wherein said vertebrate cells further comprise amplified marker gene DNA.

8. The process of claim 7 wherein said amplified marker gene [**12] DNA is Dihydrofolate reductase (DHFR) gene DNA.

9. The process according to claims 2, 4 and 6 wherein said cells are mammalian cells.

The *'080* patent, which issued with seven claims on April 15, 1997, claims both an isolated erythropoietin glycoprotein and a [*1323] method for therapeutically administering a pharmaceutical composition thereof. Only product claims 2-4 are at issue:

2. An isolated erythropoietin glycoprotein having the in vivo biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells, wherein said erythropoietin glycoprotein comprises the mature erythropoietin amino acid sequence of FIG. 6 and is not isolated from human urine.

3. A non-naturally occurring erythropoietin glycoprotein having the in vivo biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells, wherein said erythropoietin glycoprotein comprises the mature erythropoietin amino acid sequence of FIG. 6.

4. A pharmaceutical composition comprising a therapeutically effective amount of an erythropoietin glycoprotein product according to claim 1, 2, or 3.

The *'349* patent [**13] , which issued on May 26, 1998, contains one method claim and six product claims that are drawn generally to types of vertebrate cells grown in culture. At issue are claims 1, 3-4, and 6-7:

1. Vertebrate cells which can be propagated in vitro and which are capable upon growth in culture of producing erythropoietin in the medium of their growth in excess of 100 U of erythropoietin per 10<6> cells in 48 hours as determined by radioimmunoassay, said cells comprising non-human DNA sequences that control transcription of DNA encoding human erythropoietin.

3. Vertebrate cells according to claim 1 capable of producing in excess of 1000 U erythropoietin per 10<6> cells in 48 hours.

4. Vertebrate cells which can be propagated in vitro which comprise transcription control DNA sequences, other than human erythropoietin transcription control sequences, for production of human erythropoietin, and which upon growth in culture are capable of producing in the medium of their growth in excess of 100 U of erythropoietin per 10<6> cells in 48 hours as determined by radioimmunoassay

6. Vertebrate cells according to claim 4 capable of producing in excess [**14] of 1000 U erythropoietin per 10<6> cells in 48 hours.

7. A process for producing erythropoietin comprising the step of culturing, under suitable nutrient conditions, vertebrate cells according to claim 1, 2, 3, 4, 5, or 6.

n3 Because the patents in suit share an identical disclosure, all citations will be to the *'933* specification unless otherwise noted.

Last, the *'422* patent, containing two claims directed to therapeutically effective pharmaceutical compositions of EPO, was granted on September 21, 1999. Only claim 1 is in dispute:

1. A pharmaceutical composition comprising a therapeutically effective amount of human erythropoietin and a pharmaceutically acceptable diluent,

> adjuvant or carrier, wherein said erythropoietin is purified from mammalian cells grown in culture.

The district court conducted the Markman hearing in late March and early April 2000 in advance of Amgen's motion for summary judgment of infringement. The court entertained oral argument, aided by demonstrative [**15] exhibits, but heard no witness testimony and received no evidence. *Amgen, 126 F. Supp. 2d at 81, 57 USPQ2d at 1455.* At the close of the hearing, the court announced its claim constructions from the bench; these oral rulings were included and expounded upon in the written opinion ruling on the merits following trial. *Id. at 84-94, 57 USPQ2d at 1457-64.*

Immediately following the Markman hearing, the court turned to Amgen's pending motion for summary judgment of infringement of *'422* patent claim 1 and *'349* patent claims 1, 3-4, and 6. As to the *'422* patent, the district court found: (1) that it was uncontradicted that the accused [*1324] product, HMR4396, was a pharmaceutical composition; (2) that it necessarily contained a therapeutically effective amount of human erythropoietin (otherwise, the filing of an INDA would be pointless); and (3) that the record evidence demonstrated that HMR4396 contained a pharmaceutically acceptable diluent, adjuvant, or carrier as claimed in claim 1. *Id. at 94-95, 57 USPQ2d at 1455-56.* The sole remaining question was whether the accused erythropoietin product had been "purified from mammalian cells grown [**16] in culture." The court found, in light of its claim construction that the term "mammalian" comprises human cells, that the last limitation had been met. *Id. at 95-96, 57 USPQ2d at 1466.* The court therefore granted summary judgment of infringement of *'422* patent claim 1.

Trial commenced on May 15, 2000. When Amgen rested at the close of its infringement case, the court granted TKT's motions for judgment of non-infringement of the *'698* patent and literal non-infringement of the *'080* patent. *Id. at 99-104, 57 USPQ2d at 1469-73.* At the close of TKT's rebuttal case, the court granted Amgen's motion for judgment of validity, finding that TKT had not carried its burden of clearly and convincingly proving anticipation or obviousness. *Id. at 104-17, 57 USPQ2d at 1473-82.* The remaining issues were taken under advisement. The court's opinion issued on January 19, 2001, and these timely cross-appeals followed. Vested with jurisdiction under *28 U.S.C. § 1295*(a)(1), we address below the myriad issues before us.

DISCUSSION

I

The rules are by now well known. [HN1] Because claim language defines claim scope, the first step in [**17] an infringement analysis is to construe the claims, i.e., to determine the scope and meaning of that which is allegedly infringed. *Markman v. Westview Instr., Inc., 52 F.3d 967, 976, 34 USPQ2d 1321, 1326 (Fed. Cir. 1995),* aff'd, *517 U.S. 370, 38 USPQ2d 1461, 134 L. Ed. 2d 577, 116 S. Ct. 1384 (1996).* To properly construe the claims, a court must examine the claims, the rest of the specification, and, if in evidence, the prosecution history. *Vitronics Corp. v. Conceptronic, Inc., 90 F.3d 1576, 1582, 39 USPQ2d 1573, 1576-77 (Fed. Cir. 1996).* Thereafter, the properly construed claims are compared to the accused product or process to determine whether each of the claim limitations is met, either literally or equivalently. *CCS Fitness, Inc. v. Brunswick Corp., 288 F.3d 1359, 1365, 62 USPQ2d 1658, 1662 (Fed. Cir. 2002).*

There are two general areas of dispute TKT raises regarding the district court's claim construction. First, TKT urges that the court erred by failing to limit the asserted claims to exogenous DNA, despite the fact that none of the claims in suit contain an "exogenous DNA" limitation. Second, TKT asserts [**18] that the court erred by refusing to limit the terms "vertebrate," "mammalian," and "non-naturally occurring" -- each of which appear in varying degrees within the asserted claims -- such that they exclude host human cells which, of course, are used by the accused infringers. [HN2] We consider the trial court's claim construction -- a matter of law -- afresh on appellate review. See *Cybor Corp. v. FAS Tech., Inc., 138 F.3d 1448, 1455, 46 USPQ2d 1169, 1173 (Fed. Cir. 1998)* (en banc).

A

We turn first to address a threshold definitional dispute that carries with it important consequences for the infringement issues decided by the district court and facing us on appeal, to wit, what is the distinction between exogenous, as opposed to endogenous, DNA in recombinant DNA [*1325] parlance? According to TKT, it practices an innovative process using homologous recombination: it takes the ordinarily unexpressed endogenous (or "native") EPO gene in human cells and transfects "a viral promoter and certain other DNA" that does not encode EPO. That "other" DNA is inserted into the chromosome at a pre-determined, targeted location upstream from the endogenous EPO gene to produce what TKT has [**19] termed "Gene-Activated EPO," or "GA-EPO." TKT contrasts this method with that of Amgen, which TKT asserts undeniably uses exogenous DNA.

None of the asserted claims contain either an "exogenous DNA" or "endogenous DNA" limitation. n4

Based upon representations allegedly made by Amgen during the prosecution of the patents in suit, however, TKT argues that many of the claims the district court construed should have been defined narrowly to include only exogenous DNA. The district court rejected this argument, as do we.

> n4 That is not to say that there are no claims that have such a limitation. Unasserted claim 3 of the *'933* patent, for example, does contain such a limitation: "A non-naturally occurring glycoprotein product of the expression in a mammalian host cell of an exogenous DNA sequence comprising a DNA sequence encoding human erythropoietin . . . ." col. 38, lines 26-29.

[HN3] "It is the claims that measure the invention." *SRI Int'l v. Matsushita Elec. Corp., 775 F.2d 1107, 1121, 227 USPQ 577, 585 (Fed. Cir. 1985)* [**20] (en banc). Because the claims are best understood in light of the specification of which they are a part, however, courts must take extreme care when ascertaining the proper scope of the claims, lest they simultaneously import into the claims limitations that were unintended by the patentee. See, e.g., *Hoganas AB v. Dresser Indus., Inc., 9 F.3d 948, 950, 28 USPQ2d 1936, 1938 (Fed. Cir. 1993)* ("It is improper for a court to add extraneous limitations to a claim, that is limitations added wholly apart from any need to interpret what the patentee meant by particular words or phrases in the claim." (citation omitted)). The danger of improperly importing a limitation is even greater when the purported limitation is based upon a term not appearing in the claim. "If we once begin to include elements not mentioned in the claim in order to limit such claim . . ., we should never know where to stop." *Johnson Worldwide Assocs., Inc. v. Zebco Corp., 175 F.3d 985, 990, 50 USPQ2d 1607, 1610 (Fed. Cir. 1999)* (quoting *McCarty v. Lehigh Val. R.R., 160 U.S. 110, 116, 40 L. Ed. 358, 16 S. Ct. 240, 1895 Dec. Comm'r Pat. 721 (1895))*.

Amgen's inventive EPO product, according [**21] to the disclosure in the *'933* patent, is "uniquely characterized by being the product of prokaryotic or eucaryotic host expression (e.g., by bacteria, yeast and mammalian cells in culture) of exogenous DNA sequences obtained by genomic or cDNA cloning or by gene synthesis." *'933* patent, col. 10, lines 15-20. In discussing United States Patent No. *4,237,224* (issued to Cohen), the *'933* patent defines "exogenous DNA" by reference as DNA that is foreign to the host organism. See id. col. 2, lines 41-47 ("The Cohen et al. patent first involves manufacture of a transformation vector by enzymatically cleaving viral or circular plasmid DNA to form linear DNA strands. Selected foreign ('exogenous' or 'heterologous') DNA strands usually including sequences coding for desired product are prepared in linear form through use of similar enzymes."). During the prosecution of Serial No. 08/468,369, which became the *'349* patent, the examiner commented that the application "teaches and enables only cells that have been transformed with exogenous DNA that encodes erythropoietin (EPO) [*1326] that have the high EPO production required by the claims." TKT asserts, as a result, that its GA-EPO product and process [**22] fall outside the scope of the asserted claims because Amgen repeatedly has characterized its claimed products and processes as requiring the use of exogenous EPO DNA, and hence the claims should be limited thereto.

Guided by our principles of claim construction, we agree with the district court that TKT improperly seeks to import the "exogenous" limitation into the claims. The plain meaning of the claims controls here, and they plainly are not so limited. The statement that the invention is "uniquely characterized" by the expression of exogenous DNA sequences does not impel us to accept TKT's position when the asserted claims do not contain such an express limitation. In fact, TKT's position is undermined by the doctrine of claim differentiation, as reference to other claims clearly indicates that Amgen did not intend to limit the invention to the use of exogenous DNA. Unasserted claim 3 of the *'933* patent, for example, is virtually identical to claim 1, save for the express limitation regarding the use of "exogenous DNA" (underlined portioned indicating differences).

Claim 1	Claim 3
A non-naturally occurring erythropoietin glycoprotein product having the in vivo biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and having glycosylation which differs from that	A non-naturally occurring glycoprotein product of the expression in a mammalian host cells of an exogenous DNA sequence comprising a DNA sequence encoding human erythropoietin said product

Claim 1	Claim 3
of primary urinary erythropoietin.	possessing the in vivo biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and having glycosylation which differs from that of human urinary erythropoietin.

[**23]

Our court has made clear that [HN4] when a patent claim "does not contain a certain limitation and another claim does, that limitation cannot be read into the former claim in determining either validity or infringement." *SRI Int'l, 775 F.2d at 1122, 227 USPQ at 586;* see also *O.I. Corp. v. Tekmar Co., Inc., 115 F.3d 1576, 1582, 42 USPQ2d 1777, 1781 (Fed. Cir. 1997)* (expressing the notion that there are practical limits to the doctrine of claim differentiation: "the doctrine cannot alter a definition that is otherwise clear from the claim language, description, and prosecution history."). There is a rebuttable presumption that different claims are of different scope. See *Kraft Foods, Inc. v. Int'l Trading Co., 203 F.3d 1362, 1366-67, 53 USPQ2d 1814, 1817 (Fed. Cir. 2000); Multiform Dessicants, Inc. v. Medzam, Ltd., 133 F.3d 1473, 1479-80, 45 USPQ2d 1429, 1434 (Fed. Cir. 1998).*

The examiner's statement in the prosecution history gives us no pause, as the basis for his rejection was not because transformation with exogenous DNA was not taught, but because "the high EPO [*1327] production required by the claims" was not. See J. A. at 1302 [**24] ("The instant application does not guide one of ordinary skill in the art in the discovery of non-transformed vertebrate cells that are capable of the high EPO production recited in the instant claims, [as demonstrated in the reference,] each of which discloses levels of EPO production by vertebrate cells in culture that are far below those levels required in the instant claims."). TKT's position is further undermined because the asserted claims issued. We must presume the examiner did his job, and if he truly thought that the specification taught or enabled only the use of exogenous DNA, the asserted claims would not have issued.

In the end, TKT has not directed our attention to anything in the intrinsic record that rebuts the presumption that the plain meaning of the terms controls. Accordingly, we conclude that the scope of the asserted claims should not be limited to the expression of exogenous DNA.

B

TKT asserts, in addition to the exogenous/endogenous distinction discussed above, that the district court misconstrued the terms "non-naturally occurring," "vertebrate cells," and "mammalian cells" -- which appear in many of the asserted claims -- to include human [**25] cells. Reviving the same argument the district court rejected below, TKT contends Amgen expressly disavowed the use of human cells to make human EPO.

The district court found that the definition of the term "non-naturally occurring" can be discerned through the doctrine of claim differentiation. Specifically, the court concluded that TKT's proffered construction must fail in light of *'933* patent claim 3, discussed previously, which claims a "non-naturally occurring glycoprotein product of the expression in a mammalian host cell of an exogenous DNA sequence encoding human erythropoietin . . . ." By its terms, then, this claim would cover the expression of human DNA in a cat host cell, for example, because a cat is a mammal. The court thus concluded that the phrase "non-naturally occurring" would be redundant in claim 3 if the phrase had the meaning TKT sought to ascribe to it. Further, because the patent specification compares the biological activity of synthetic products to "EPO isolates from natural sources" or "natural EPO isolates," the court concluded that non-naturally occurring simply means "not occurring in nature." *Amgen, 126 F. Supp. 2d at 90-91, 57 USPQ2d at 1462-63.* [**26]

Similarly, finding that the term vertebrate is widely known and understood to cover anything with "a segmented bony or cartilaginous spinal cord [which obviously includes humans]," *id. at 85, 57 USPQ2d at 1457-58,* the court adopted Amgen's proposed construction. The court also adopted Amgen's proposed construction of the term "mammalian cells" appearing in *'422* patent claim 1 and *'698* patent claim 9 under a similar rationale. *Id. at 84-86, 57 USPQ2d at 1458.*

[HN5] We indulge a heavy presumption that a claim term carries its ordinary and customary meaning. *CCS Fitness, 288 F.3d at 1366, 62 USPQ2d at 1662;* see also *Gart v. Logitech, Inc., 254 F.3d 1334, 1341, 59 USPQ2d 1290, 1295 (Fed. Cir. 2001).* Although TKT is correct

that the prosecution history is always relevant to claim construction, it is also true that the prosecution history may not be used to infer the intentional narrowing of a claim absent the applicant's clear disavowal of claim coverage, such as an amendment to overcome a rejection. See *York Prods., Inc. v. Central Tractor & Farm Fam. Ctr., 99 F.3d 1568, 1575, 40 USPQ2d 1619, 1624 (Fed. Cir. 1996).* [**27] No such clear disavowal occurred here.

[*1328] We agree with Amgen that the specification expressly describes humans as a subset of mammals, and mammals, in turn, as a subset of vertebrates. See *'933* patent, col. 4, lines 47-48; col. 10, line 21. Moreover, the specification can fairly be read to, if not expressly, disclose the use of human DNA in human host cells in culture:

> Conspicuously comprehended are expression systems involving vectors of homogeneous origins applied to a variety of bacterial, yeast, and mammalian cells in culture as well as to expression systems not involving vectors . . . . In this regard, it will be understood that expression of, e.g., monkey origin DNA in monkey host cells in culture and human host cells in culture, actually constitute instances of 'exogenous' DNA expression inasmuch as the EPO DNA whose high level expression is sought would not have its origins in the genome of the host.

'933 patent, col. 37, lines 33-43 (emphasis added). The astute reader will observe what appears to be a breakdown in the parallelism of the sentence emphasized in the block quote above. Specifically, the reference to the expression of "monkey origin DNA [**28] in monkey host cells in culture and human host cells in culture" seems a bit nonsensical because the expression of monkey origin DNA in human host cells is perforce the expression of exogenous DNA. The original 1983 application from which all the patents in suit claim priority, by contrast, contained language that upholds the parallelism of the sentence and logically makes sense. It read, in pertinent part: "It will be understood that expression of, e.g., monkey origin DNA in monkey host cells in culture and human DNA in human host cells in culture constitute instances of 'exogenous' DNA expression." J.A. at 2862 (emphasis added).

TKT boldly asserts that the variance between the original application and the patents in suit bespeaks some volitional act by Amgen to narrow the scope of the asserted claims in light of certain experimental data. In particular, TKT advances a theory whereby Amgen intentionally removed the language from subsequent applications (allegedly) because test results using human cells were not good, and later admitted (during an opposition proceeding against the European counterpart patent) that the omission was not inadvertent. But the record contains a more [**29] benign explanation as to what happened. According to the testimony of Dr. Lin, he was unaware of, and therefore did not authorize, the change. Further, the prosecuting attorney testified in his deposition that to the best of his knowledge the error was a typographical error.

But even assuming that the error was intentional, the district court's claim construction would not be foreclosed: our precedent is clear that [HN6] claims are not perforce limited to the embodiments disclosed in the specification. E.g., *Rexnord Corp. v. Laitram Corp., 274 F.3d 1336, 1344, 60 USPQ2d 1851, 1856 (Fed. Cir. 2001)* ("An applicant is not required to describe in the specification every conceivable and possible future embodiment of his invention."). Here, the patent plainly discloses the use of human host cells in culture, and our review of the record indicates no "clear disavowal" sufficient to undercut the express disclosure in the specification.

As a result, we are satisfied that the terms "non-naturally occurring," "vertebrate," and "mammalian" should be construed as they were by the district court, in a manner consistent with their plain meaning. Accordingly, we reject TKT's attempt [**30] to limit the scope of the asserted claims under an unduly constricted reading of the specification.

C

The final claim construction issue TKT raises is aimed at the district court's [*1329] alleged failure to discern "source and process" limitations in claims of the *'080*, *'349*, and *'422* patents. According to TKT, the trial court erred by concluding that the asserted claims are product claims, i.e., that they are directed to a structural entity that is not defined or limited by how it is made. TKT summarily states that this holding must be erroneous because, it asserts, the patentability of the claims depended on the process since "Amgen tried, but failed, to distinguish rEPO from prior art EPOs based on physical differences." We do not agree.

It is telling that neither in the briefing nor at oral argument did TKT direct us to any specific statement in the prosecution history to support the contention that the patentability of the product claims in suit depended upon

the process by which those products are obtained. In fact, the original claims of at least one of the patents (the *'080* patent) were drafted as product-by-process claims, which claims were cancelled and replaced with "pure" [**31] product claims. This is strong evidence that both the patentee and the examiner viewed the claims that ultimately issued as lacking a process component. See *Vanguard Prods., Inc. v. Parker Hannifin Corp., 234 F.3d 1370, 1372, 57 USPQ2d 1087, 1089 (Fed. Cir. 2000)* ("Parker Hannifin argues that the prosecution history shows that the Vanguard inventors viewed co-extrusion as 'fundamental' to manufacture of the claimed gasket, thereby imposing this process of manufacture upon the product claims . . . . However, review of the prosecution history shows that during examination the examiner as well as the applicant treated the product claims as directed to the product itself, and examined the application accordingly.").

In any event, we are not convinced that the source limitations in the asserted claims convert the claims into anything other than product claims. As to the *'080* patent, the "non-naturally occurring" limitation in claims 3 and 4 merely prevents Amgen from claiming the human EPO produced in the natural course. By limiting its claims in this way Amgen simply avoids claiming specific subject matter that would be unpatentable under § 101. This court has endorsed [**32] this approach, recognizing that [HN7] patentees can use negative limitations such as "non-human" and "non-natural" to avoid rejection under § 101. See *Animal Legal Def. Fund v. Quigg, 932 F.2d 920, 923, 18 USPQ2d 1677, 1680 (Fed. Cir. 1991).* The district court arrived at a similar conclusion, *Amgen, 126 F. Supp. 2d at 89, 57 USPQ2d at 1462-63,* and TKT has not demonstrated any error in that conclusion. Similarly, the "not isolated from human urine" limitation in claims 2 and 4 of the *'080* patent simply requires that the claimed EPO, however made, be obtained from a source other than human urine. Each of these limitations only excludes human EPO from specific sources and does not restrict the claimed EPO to that produced from any particular source or by any particular method. In sum, claims 2, 3, and 4 of the *'080* patent remain broadly drawn to the described "erythropoietin glycoprotein" or "pharmaceutical composition" produced by any method, or obtained from any source, other than those specifically excluded.

As to the *'422* patent, the limitation "purified from mammalian cells grown in culture" in claim 1 clearly limits the source of the EPO used in [**33] the claimed "pharmaceutical composition." The limitation only speaks to the source of the EPO and does not limit the process by which the EPO is expressed. Rather, the claim is broadly drawn to a "pharmaceutical composition" having certain elements, one of those being EPO "purified from mammalian cells in culture." This reading is in line with the [*1330] district court's construction and, again, TKT directs us to no error. n5

n5 We do not hold that these limitations lack meaning, only that they mean just what they say. Accordingly, they limit only the source from which the EPO is obtained, not the method by which it is produced.

II

[HN8] It is axiomatic that claims are construed the same way for both invalidity and infringement. *W.L. Gore & Assoc., Inc. v. Garlock, Inc., 842 F.2d 1275, 1279, 6 USPQ2d 1277, 1280 (Fed. Cir. 1988).* But because the features of the accused product or process are often undisputed, this axiom invites a common approach in the appellate arguments by accused infringers: the principal [**34] argument challenges the correctness of a trial court's broad claim construction; the contingent argument, assuming the trial court's claim construction is affirmed, challenges validity under *35 U.S.C. § 112* P 1 of the asserted patents in light of that broad construction. See, e.g., *Adv. Cardiovascular Sys. v. Medtronic, Inc., 265 F.3d 1294, 60 USPQ2d 1161 (Fed. Cir. 2001); PPG Indus. v. Guardian Indus. Corp., 75 F.3d 1558, 37 USPQ2d 1618 (Fed. Cir. 1996); Kalman v. Kimberly-Clark Corp., 713 F.2d 760, 218 USPQ 781 (Fed. Cir. 1983).* TKT employs that approach here. We therefore think it appropriate to address the relevant § 112 issues before turning to the issue of infringement.

[HN9] Section 112 of the patent statute describes what must be contained in the patent specification. Among other things, it must contain "a written description of the invention, and of the manner and process of making and using it . . . [such] as to enable any person of ordinary skill in the art to which it pertains . . . to make and use the same . . . ." *35 U.S.C. § 112* P 1. Thus, this statutory language mandates [**35] satisfaction of two separate and independent requirements: an applicant must both describe the claimed invention adequately and enable its reproduction and use. See *Vas-Cath Inc. v. Mahurkar, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991).* Third, though not in issue here, he must disclose what he considers the best mode of practicing his invention.

A

[HN10] The purpose of the written description requirement is to prevent an applicant from later asserting that he invented that which he did not; the applicant for a patent is therefore required to "recount his invention in such detail that his future claims can be

determined to be encompassed within his original creation." *Id. at 1561, 19 USPQ2d at 1115* (citation omitted). Satisfaction of this requirement is measured by the understanding of the ordinarily skilled artisan. *Lockwood v. Am. Airlines, Inc., 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997)* ("The description must clearly allow persons of ordinary skill in the art to recognize that [the inventor] invented what is claimed."). "Compliance with the written description requirement is essentially a fact-based inquiry [**36] that will 'necessarily vary depending on the nature of the invention claimed.'" *Enzo Biochem v. Gen-Probe, Inc., 296 F.3d 1316, 1324, 63 USPQ2d 1609, 1613 (Fed. Cir. 2002)* (citation omitted). Because of its fact intensive nature, we review a district court's decision on the adequacy of written description for clear error. *Purdue Pharma L.P. v. Faulding Inc., 230 F.3d 1320, 1323, 56 USPQ2d 1481, 1483 (Fed. Cir. 2000)* (citations omitted).

In addressing TKT's written description arguments, the district court carefully examined whether Amgen's specification adequately described the full breadth of the claims. [*1331] In the end, the district court rejected TKT's written description challenge, finding that TKT had proven its case only by a preponderance of the evidence -- not the clear and convincing standard required as a matter of law. Acknowledging the presence of "a genuine dispute between the expert witnesses," the court weighed the testimony and found that the evidence showed that the descriptions adequately described to those of ordinary skill in the art in 1984 the use of the broad class of available mammalian and vertebrate cells to produce the claimed [**37] high levels of human EPO in culture. *Amgen, 126 F. Supp. 2d at 149, 57 USPQ2d at 1507.* In so doing, the court credited in particular the testimony of Amgen's expert, Dr. Harvey Lodish, who testified, among other things, that there might be "minor differences" in applying the method of the disclosed examples (utilizing CHO and COS-1 (monkey) cells) to any vertebrate or mammalian cells, but that those of ordinary skill could "easily" figure out those differences in methodology. *Id., 57 USPQ2d at 1507.*

Much of TKT's argument on appeal challenging this finding dovetails with its claim construction arguments we have already found lacking. For example, TKT asserts that the Amgen patents do not satisfy the written description requirement because: (1) Amgen failed to sufficiently describe the use of all vertebrate and mammalian cells; (2) Amgen deleted use of exogenous human EPO DNA in human cells from its applications; n6 (3) Amgen expressly excluded the use of endogenous EPO DNA; (4) Amgen emphasized that the advantage of its invention was "freedom from association with human proteins"; and (5) in using the "uniquely characterized" language to describe [**38] the polypeptides of the invention, Amgen identified exogenous EPO DNA as an essential element of the invention. As a result of these shortcomings, argues TKT, it has clearly and convincingly proven invalidity under *Regents of the University of California v. Eli Lilly & Co., 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997), Gentry Gallery, Inc. v. Berkline Corp., 134 F.3d 1473, 45 USPQ2d 1498 (Fed. Cir. 1998),* and *Enzo Biochem, Inc. v. Gen-Probe, Inc., 296 F.3d 1316, 63 USPQ2d 1609 (Fed. Cir. 2002).* We are not persuaded that these precedents mandate reversal of the trial court's factual findings as clearly erroneous regarding the written descriptions.

> n6 We addressed this point in our claim construction analysis on pages 17-18 ante, finding that the written description did not exclude human cells from the scope of the claims. That analysis suffices here as well.

First, in addressing the adequacy of the written description of the *'422* patent and with respect [**39] to TKT's exogenous DNA arguments, the district court noted:

> When the claim is to a composition rather than a process, the written description requirement does not demand that the specification describe technological developments in the way in which the claimed composition is made that may arise after the patent application is filed. See *United States Steel Corp. v. Phillips Petroleum Co., 865 F.2d 1247, 1251 [9 USPQ2d 1461, 1465]* (Fed. Cir. 1989); *In re Koller, 613 F.2d 819, 824-25 [204 USPQ 702, 707]* (Fed. Cir. 1980); see also *In re Hogan, 559 F.2d 595, 606 [194 USPQ 527, 538]* (C.C.P.A. 1977). Instead, section 112 only requires the Court to determine whether the specification conveys to one of ordinary skill in the art as of 1984 that Dr. Lin invented the subject matter claimed in the patents-in-suit. *Reiffin, 214 F.3d at 1346 [Reiffin v. Microsoft Corp., 214 F.3d 1342, 1346, 54 USPQ2d 1915, 1917 (Fed. Cir. 2000)].* The written description inquiry, [*1332] therefore, focuses on a comparison between the specification and the invention referenced by the terms of

> the claim -- not comparison between [**40] how the product was made as disclosed in the patent and future developments of this process that might alter or even improve how the same product is made.

Amgen, 126 F. Supp. 2d at 150, 57 USPQ2d at 1508; see also *id. at 152, 57 USPQ2d at 1509* (discussing the *'080* patent), 154 n.51, *57 USPQ2d at 1510* (discussing the *'349* patent). The district court therefore considered TKT's exogenous DNA arguments and, for the reasons stated above, rejected them. On appeal TKT has not argued that its legal analysis was erroneous. Because we have not been directed to any case law to the contrary, we conclude the district court's legal conclusion based on Phillips Petroleum was not erroneous and that it properly handled the exogenous DNA issue.

We move now to TKT's argument that Amgen failed to sufficiently describe all vertebrate and mammalian cells as engineered in the claimed invention. We held in Eli Lilly that the adequate description of claimed DNA requires a precise definition of the DNA sequence itself -- not merely a recitation of its function or a reference to a potential method for isolating it. *119 F.3d at 1566-67, 43 USPQ2d at 1406* [**41] (holding the disclosure of the cDNA sequence of the insulin gene of a rat did not adequately describe the cDNA sequence of the insulin gene of every vertebrate). More recently, in Enzo Biochem, we clarified that Eli Lilly did not hold that all functional descriptions of genetic material necessarily fail as a matter of law to meet the written description requirement; rather, the requirement may be satisfied if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure. See *Enzo Biochem, 296 F.3d at 1324, 63 USPQ2d at 1613.* Both Eli Lilly and Enzo Biochem are inapposite to this case because the claim terms at issue here are not new or unknown biological materials that ordinarily skilled artisans would easily miscomprehend. n7 Instead, the claims of Amgen's patents refer to types of cells that can be used to produce recombinant human EPO. Thus, TKT can only challenge the adequacy of disclosure of the vertebrate or mammalian host cell -- not the human DNA itself. This difference alone sufficiently distinguishes Eli Lilly, because when used, as here, merely to identify types of cells (instead of undescribed, [**42] previously unknown DNA sequences), the words "vertebrate" and "mammalian" readily "convey[] distinguishing information concerning [their] identity" such that one of ordinary skill in the art could "visualize or recognize the identity of the members of the genus." *Eli Lilly, 119 F.3d at 1567, 1568, 43 USPQ2d at 1406.* n8 Indeed, the district court's reasoned conclusion that the specification's description of producing the claimed EPO in two species of vertebrate or mammalian cells adequately supports claims covering EPO made using the genus vertebrate or mammalian cells, renders Eli Lilly listless in this case. *Amgen, 126 F. Supp. 2d at 149, 57 USPQ2d at 1507.*

n7 Indeed, Amgen's patents appear to satisfy the sequence requirement in Eli Lilly insofar as Figure 6 of the patents expressly discloses the complete (albeit slightly incorrect) sequence of human genomic EPO DNA and the encoded DNA.

n8 There is no issue here as to in haec verba description because, as stated in the body of the opinion, in contrast to "cDNA" -- that clearly does not describe the actual sequence of the cDNA -- the words "mammalian cells" and "vertebrate cells" convey exactly what they are. Thus, this aspect of the holding in Eli Lilly is also inapplicable here.

[**43]

[*1333] TKT's remaining arguments rely on Gentry Gallery. However, we see Gentry Gallery as similarly inapt. TKT would have us view Gentry as a watershed case, in reliance on an isolated statement -- probably only dicta -- that one of ordinary skill in the art would clearly understand that the location of the reclining controls on the claimed sectional sofa "was not only important, but essential to [the] invention." *134 F.3d at 1480, 45 USPQ2d at 1503.* But as we recently indicated in *Cooper Cameron Corp. v. Kvaerner Oilfield Prods., Inc., 291 F.3d 1317, 1323, 62 USPQ2d 1846, 1850-51 (Fed. Cir. 2002),* "we did not announce [in Gentry] a new 'essential element' test mandating an inquiry into what an inventor considers to be essential to his invention and requiring that the claims incorporate those elements." See also *Vas-Cath, 935 F.2d at 1565, 19 USPQ2d at 1114;* cf. *Aro Mfg. Co. v. Convertible Top Replacement Co., 365 U.S. 336, 345, 5 L. Ed. 2d 592, 81 S. Ct. 599, 1961 Dec. Comm'r Pat. 635 (1961)* ("There is no legally recognizable or protected 'essential element,' 'gist' or 'heart' of the invention in a combination patent."). Understood [**44] in this light, one sees the holding in Gentry for what it really was: an application of the settled principle that [HN11] a broadly drafted claim must be fully supported by the written description and drawings. See *Cooper Cameron, 291 F.3d at 1323, 62 USPQ2d at 1850-51.* After considering extensive testimony from both parties, the district court held this principle met and TKT failed to demonstrate that this analysis was clearly erroneous factually or based on an

error of law. *Amgen, 126 F. Supp. 2d at 149-50, 57 USPQ2d at 1507-08.*

To the extent the particular facts of Gentry are relevant, we also find it distinguishable. First, there is a fundamental difference between Amgen's patented invention and the invention in Gentry. In Gentry the invention was the placement of reclining controls on a central console on a unit of a sectional sofa so as to allow the sofa to have two independent reclining seats face in the same direction (solving a problem present in the prior art). *134 F.3d at 1475, 45 USPQ2d at 1499.* The undisclosed element leading to the Gentry court's holding of invalidity for lack of an adequate description [**45] was a location for the controls other than on the console -- leading to a different and undescribed product. See *id. at 1479, 45 USPQ2d at 1502-03.* Amgen's invention is not the location of the control sequences and EPO DNA in relation to the cell, but rather the production of human EPO using those sequences. Thus, the undisclosed element TKT urges invalidates Amgen's product claims is a different method (endogenous activation) of making the claimed compositions. But, as the district court noted, under our precedent [HN12] the patentee need only describe the invention as claimed, and need not describe an unclaimed method of making the claimed product. *Amgen, 126 F. Supp. 2d at 150, 57 USPQ2d at 1507* (citing *Phillips Petroleum, 865 F.2d at 1251, 9 USPQ2d at 1465; In re Koller, 613 F.2d at 824-25, 204 USPQ at 707);* see also *Vas-Cath, 935 F.2d at 1563-64, 19 USPQ2d at 1117.* This factual difference alone is sufficient to distinguish this case from Gentry.

Second, the statements by the patentee in the written description in this case fall short of what Gentry prohibits. The court in Gentry [**46] concluded that the inventor had clearly expressed in the written description that he considered his invention to be limited to the specific location of the controls on the console on the sofa ("the only possible location") and that any variation was "outside the stated purpose of the invention." *Gentry Gallery, 134 F.3d at 1479, 45 USPQ2d at 1503.* Indeed, in Gentry the inventor testified that he only considered locating the controls outside of the console -- and only broadened his application claims accordingly -- after seeing Gentry's competitors introduce products with [*1334] controls located off the console. Id. Here, to be sure, Amgen made statements that its invention is "uniquely characterized" by exogenous expression of DNA. *'933* patent col. 10, lines 15-20. When considered in context, however, these statements do not lead to the same conclusion as in Gentry. Amgen's statements simply do not clearly indicate that exogenous expression is the only possible mode of the invention or that other methods were outside the stated purpose of the invention. Instead, Amgen begins the background section of its written description by stating "the present invention [**47] relates generally to the manipulation of genetic materials and, more particularly, to recombinant procedures making possible the production of polypeptides possessing part or all of the primary structural conformation and/or one or more of the biological properties of naturally occurring erythropoietin." *'933* Patent, col. 1, lines 18-23. Because of this lack of clear statements by the patentee limiting the claimed invention (and in light of the case law discussed, ante), we cannot invalidate a patent for failure to describe a method of producing the claimed compositions that is not itself claimed. Nor could the patentee have described the other method, as it was not developed until 10 years later. We see Gentry Gallery as inapplicable in this regard. In light of the evidentiary record and TKT's inability to persuade us that precedent requires a contrary result, we hold that the district court's finding that Amgen satisfied the written description requirement is not clearly erroneous.

B

[HN13] The enablement requirement is often more indulgent than the written description requirement. The specification need not explicitly teach those in the art to make and use the invention; the [**48] requirement is satisfied if, given what they already know, the specification teaches those in the art enough that they can make and use the invention without "undue experimentation." *Genentech, Inc. v. Novo Nordisk, A/S, 108 F.3d 1361, 1365, 42 USPQ2d 1001, 1004 (Fed. Cir. 1997); In re Vaeck, 947 F.2d 488, 495, 20 USPQ2d 1438, 1444 (Fed. Cir. 1991).* Before the district court, TKT bore the burden of clearly and convincingly proving facts showing that the claims were not enabled. E.g., *Enzo Biochem, Inc. v. Calgene, Inc., 188 F.3d 1362, 1375, 52 USPQ2d 1129, 1141 (Fed. Cir. 1999).* Enablement is a question of law; we therefore review the trial court's determination de novo, deferring to its assessment of subsidiary facts underlying the legal question unless clearly erroneous. *Bruning v. Hirose, 161 F.3d 681, 686, 48 USPQ2d 1934, 1939 (Fed. Cir. 1998).*

TKT contends that the asserted claims are invalid for lack of enablement. Taking a position that virtually mirrors the written description (and claim construction) arguments previously rejected, TKT posits that the specifications do not enable an ordinarily skilled [**49] artisan to practice the full scope of the asserted claims without undue experimentation because they fail to describe the production of EPO using human cells or endogenous human EPO DNA. At bottom, TKT complains that the court erred by failing to follow its findings to their logical conclusion. n9

n9 TKT refers here to the district court's statement that "it appears that Dr. Lin claimed far more than he delivered." *Amgen, 126 F. Supp. 2d at 158, 57 USPQ2d at 1514.* Although this statement does seem out of kilter with the court's ultimate holding, we understand it in light of how close the court viewed the issue: "After much reflection, the court finds that Amgen survives [the enablement challenge], albeit barely." *Id. at 157, 57 USPQ2d at 1513.*

But the district court made thorough and complete factual findings [*1335] supporting its holding that the claims were not proven not enabled, expressly incorporating many of its factual determinations made with respect to written description. [**50] As to TKT's endogenous/exogenous arguments, the court concluded the arguments were inapplicable as a matter of law for two reasons. First, [HN14] "where the method is immaterial to the claim, the enablement inquiry simply does not require the specification to describe technological developments concerning the method by which a patented composition is made that may arise after the patent application is filed." *Amgen, 126 F. Supp. 2d at 160, 57 USPQ2d at 1515* (citing *Phillips Petroleum, 865 F.2d at 1251, 9 USPQ2d at 1465; In re Koller, 613 F.2d at 824-25, 204 USPQ at 707; In re Hogan, 559 F.2d at 606, 194 USPQ at 538);* id. at 161, 57 USPQ2d at 1516 (discussing the *'080* patent), 163-64, *57 USPQ2d at 1518* (discussing the *'349* patent). Thus, the specification's failure to disclose the later-developed endogenous activation technology cannot invalidate the patent. *Id. at 160, 57 USPQ2d at 1516.* Second, [HN15] "the law makes clear that the specification need teach only one mode of making and using a claimed composition." *Id. at 160, 57 USPQ2d at 1515* (citing *Johns Hopkins Univ. v. Cellpro, Inc., 152 F.3d 1342, 1361, 47 USPQ2d 1705, 1719 (Fed. Cir. 1998);* [**51] *Engel Indus. Inc. v. Lockformer Co., 946 F.2d 1528, 1533, 20 USPQ2d 1300, 1304 (Fed. Cir. 1991));* see also *Durel Corp. v. Osram Sylvania Inc., 256 F.3d 1298, 1308, 59 USPQ2d 1238, 1244 (Fed. Cir. 2001).* This conclusion again makes the specification's failure to disclose TKT's endogenous activation technology legally irrelevant. *Amgen, 126 F. Supp. 2d at 160, 57 USPQ2d at 1515.* We reach the same conclusion on appeal, as TKT has not persuaded us that the district court's conclusions in this regard were erroneous.

Focusing specifically on the *'422* patent, the enablement inquiry is whether Amgen has enabled all pharmaceutical compositions comprising "a therapeutically effective amount of human erythropoietin," "a pharmaceutically acceptable diluent, adjuvant or carrier," and human erythropoietin "purified from mammalian cells grown in culture." The court found that the specification described and enabled various possible diluents and carriers and provided specific information on effective dosages and therapeutic effect in mice. *Id. at 148, 57 USPQ2d at 1506.* Amgen also described and enabled at least one way of obtaining [**52] EPO purified from mammalian cells in culture: the genetic manipulation of CHO and COS-1 cells, followed by both described and other well known purification techniques. Finally, the court accepted testimony indicating that an ordinarily skilled artisan would infer from the COS-1 (monkey) and CHO cell examples that similar outcomes could be expected from other mammalian cells since all mammalian cells produce and secrete hormones like EPO by means of the same fundamental processes. *Id. at 159, 57 USPQ2d at 1514-15.* These are all findings of fact and they have not been shown to be clearly erroneous.

As to the *'080* patent, the inquiry is whether Amgen has enabled the production of all EPO glycoproteins having "the in vivo biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells," "the mature erythropoietin amino acid sequence of FIG. 6," and "[are] not isolated from human urine" or "non-naturally occurring." The court noted that Amgen disclosed the in vivo biological effect of EPO upon hematocrit levels in mice and adequately disclosed the sequence of the amino acid residues [*1336] in figure 6. *Id. at 151, 57 USPQ2d at 1508-09.* [**53] Amgen also described and enabled at least one method of producing EPO that was both "non-naturally occurring" and "not isolated from human urine": the genetic manipulation of CHO and COS-1 cells. The court noted with particularity that even TKT's witness, Dr. Kingston, agreed that if one of ordinary skill in the art followed the teachings of Example 10, then such a person could successfully practice the claimed invention. *Id. at 161, 57 USPQ2d at 1516.*

We address the product claims of the *'349* patent in more detail, as they differ slightly from the patents we discussed above. The *'349* patent claims genetically manipulated "vertebrate cells" -- a composition -- having certain characteristics and properties, including an ability to produce the claimed levels of human EPO. n10 The enablement question thus posed is this: having disclosed one way to make the claimed EPO-producing cell, is Amgen entitled to claim all such cells that "can be propagated in vitro," comprise "non-human DNA sequences that control transcription," transcribe "DNA encoding human erythropoietin," and produce the claimed amount of EPO? While our precedent does hold that disclosure of one or two [**54] species may not enable a broad genus, e.g., *In re Vaeck, 947 F.2d at 495-*

96, 20 USPQ2d at 1444-45, the district court made several fact-findings indicating that any gaps between the disclosures and the claim breadth could be easily bridged. See, e.g., *Amgen, 126 F. Supp. 2d at 149, 57 USPQ2d at 1514* (crediting Amgen's expert Dr. Lodish's statement that "one of ordinary skill in the art, me, my students, would have understood this not to be limited to the specific types of cells that were used in this example, that other vertebrate cells, mammalian cells, could have been used"); cf. *Enzo Biochem, 188 F.3d at 1367-68, 1372, 52 USPQ2d at 1133, 1136-37* (affirming nonenablement of claims to anti-sense DNA technology applied to all eukaryotic and prokaryotic organisms because anti-sense was a "highly unpredictable technology" and a "high quantity of experimentation" would be needed to practice the invention outside of the disclosed example); *Vaeck, 947 F.2d at 495-96, 20 USPQ2d at 1444-45* (holding the examiner did not err in rejecting as nonenabled claims drawn to all genetically-engineered cyanobacteria expressing [**55] a given protein because the claimed 150 genera of cyanobacteria represent a vast, diverse, and poorly understood group; heterologous gene expression in cyanobacteria was "unpredictable"; and the patent's disclosure referred to only a genus). The district court found that a skilled artisan could readily have used various cultured vertebrate and mammalian cells to produce human EPO, and this fact was buttressed by numerous post-filing publications that demonstrated the extent of the enabling disclosure. *Amgen, 126 F. Supp. 2d at 162, 57 USPQ2d at 1517* (citing *Gould v. Quigg, 822 F.2d 1074, 3 U.S.P.Q.2D (BNA) 1302 (Fed. Cir. 1987)* for the proposition that an expert may rely on post-filing publications to show enablement). The court also found that for those skilled in the art it was a relatively simple matter to determine whether a certain promoter would work within a specific vertebrate cell, whether a particular vertebrate cell would produce human EPO in culture, and whether a [*1337] particular promoter could be operatively linked to control the transcription of the human EPO DNA. Id. In summary, the court once again chose to credit Amgen's witnesses, [**56] Drs. Lodish and Wall, on the issue of enablement:

> Throughout the testimony of these witnesses, a theme becomes apparent: any challenge which one of ordinary skill in 1984 might have encountered in attempting to make and use the claimed invention using other cultured mammalian cells could be resolved by experimentation falling short of undue.

Id. at 159, 57 USPQ2d at 1515.

> n10 Following the dissent's "machine" analogy, the "machine" is a genetically altered vertebrate cell containing transcription control sequences used to transcribe a human EPO gene to express the claimed levels of human EPO. Simply altering the way the human EPO gene is inserted or activated -- whether it be through transformation with exogenous DNA or through activation of an endogenous gene -- does not make this a different "machine" once built; rather, it only changes the way it was "constructed."

With these factual findings before us, TKT cannot prevail simply by reasserting in a conclusory manner that Amgen's disclosure [**57] does not enable the transformation of all mammalian or vertebrate cells or the production of human EPO. The district court carefully considered these issues, finding in the end that TKT had not met its clear and convincing burden of proof. Finding no clear error in these factual determinations, and having been directed to no legal error committed by the trial court, we will not disturb its holding that the asserted patents are not invalid for failure to meet the enablement requirement of § *112 P 1.*

C

Certain concerns are raised by the dissent. My brother in dissent sees the district court as having "abstained from fully inquiring" about compliance with the written description and enablement requirements of § 112, P 1. In light of this strong statement, we write here to highlight what the district court did and did not do in deciding the case below. The district court should be seen as deciding the challenges to validity under each requirement as presented to it by the accused infringer. In doing so, the court fully found the facts that undergirded its conclusions on validity and relied on our case law interpreting and applying § 112. [HN16] We are largely limited on review [**58] to deciding whether those findings based on that testimony are clearly erroneous and we cannot so conclude. We may, of course, review de novo the court's interpretation of our precedent.

The dissent, however, does not directly challenge the court's factual findings, nor does it mention the decisions relied on by the district court. Instead, it finds fault in the absence of discussion of other precedents, namely Eli Lilly, Gentry Gallery, In re Mayhew, and In re Vaeck, and makes broader arguments seemingly based upon policy considerations.

The dissent would vacate and remand the written description issue because the district court did not cite our precedents Eli Lilly and Gentry Gallery. According to the dissent, the district court "did not focus on the

correct law to be applied" and, for that reason, its "factual findings merit no deference." It is difficult to see how the district court's analysis must be rejected because it did not include discussions of these two decisions or, per the dissent, "the principles they espouse." First, it is far from clear that the defendant based its written description challenge below primarily on these two cases. Second, [**59] as we hold above, these cases are simply inapplicable here. Given these considerations, we decline to hold that the failure of the district court to cite these precedents constitutes reversible error.

In addressing the enablement inquiry the dissent looks to two other cases not discussed by the district court. It cites *In re Mayhew, 527 F.2d 1229, 1233, 188 USPQ 356, 358 (C.C.P.A. 1976),* for the proposition that "claims failing to recite a necessary element of the invention fail for lack of an enabling disclosure." There, however, the method claims omitted a step without which the invention as claimed was [*1338] wholly inoperative (meaning it simply would not work and could not produce the claimed product). Id. Here, the lack of a limitation directed to the exogenous expression vector in the product claims is not a failure to describe the structure of the cell or a necessary element of the claimed EPO. Once inside the cell, the transcription control sequence and the human EPO DNA integrate randomly into the host cell chromosomes. The only required description, then, is of the EPO DNA and the transcription control sequences because it is the presence of these sequences [**60] in the cell that causes the cell to produce the EPO as claimed. Thus, the lack of a description of (or a limitation directed to) the expression vector itself (as separate from the EPO DNA and transcription control sequences) does not render the invention inoperable and therefore does not run afoul of *In re Mayhew, 527 F.2d at 1233, 188 USPQ at 358* (affirming examiner's rejection of claims not limited to having a cooling zone at the exit of a steel strip from a zinc bath because the specification indicated that without that cooling bath the invented process would not work).

The dissent's reliance on In re Vaeck is also misplaced. Vaeck is cited for the proposition that the disclosure of one or two species (here monkey and hamster cells) "may not enable a broad genus under the circumstances." *947 F.2d at 496, 20 USPQ2d at 1444-45.* But then again, it may; the inquiry is fact-specific. Here the district court held the disclosure did enable the genus because the differences between using the two described mammalian (and vertebrate) cells and other such cells were small and easily accommodated by the artisan. Thus, in assessing the evidence, the court [**61] found that the defendant's evidence fell short of clear and convincing.

But more fundamentally, we think the dissent unfairly characterizes the district court's careful and reasoned handling of the § 112 issues. The dissent repeatedly suggests that the district court "simply refused" to consider whether, having disclosed only one means to make EPO produced by vertebrate or mammalian cells, Amgen was entitled to claims for all such cells and EPO. Specifically, the dissent asserts that the district court "abstained" from considering whether the absence of a claim limitation on the means of expression raises § 112 issues. n11 We find this hard to understand. The district court explicitly analyzed these requirements in addressing defendant's specific challenges to validity. It decided they were not proven sufficiently and its decision is supported both by citations to our precedent and its own factual findings. Thus, rather than refusing to answer the § 112 questions, it seems the district court did answer them affirmatively.

n11 In this same vein, the dissent suggests that our court here has somehow "waived" the requirements of § 112 for product claims.

[**62]

In addressing this specific issue, the district court relied principally on two of our precedents: Phillips Petroleum and Cellpro. The court construed the former as not requiring the written description to include later-developed methods for making a claimed product. *Amgen, 126 F. Supp. 2d at 150, 160, 57 USPQ2d at 1508, 1515.* The court construed the latter as holding that a product claim is supported by adequate written description and enabling disclosure even if it describes only one method of making the claimed product. *Id. at 160, 57 USPQ2d at 1515.* These cases have not been shown to be incorrectly applied by the district court. And we, like the district court, are obligated to follow them both, as they explicitly support the court's rulings. *Phillips Petroleum,* [*1339] *865 F.2d at 1251, 9 USPQ2d at 1465* (holding that the patentee was entitled to a prior filing date because the earlier disclosure of polypropylene as known at that time described and enabled a later claim to "normally solid polypropylene" even though a new, higher molecular weight form of polypropylene had been subsequently discovered), and *Cellpro, 152 F.3d at 1361, 47 USPQ2d at 1719* [**63] (affirming summary judgment of enablement of a product claim over a challenge that two alternative embodiments disclosed in the patent were not enabled because "the enablement requirement is met if the description enables any mode of making and using the invention").

Rather than addressing these precedents, the dissent makes broad arguments that are not specifically grounded in our precedent. The dissent asks whether Amgen's disclosure "entitles it to claim all EPO produced by mammalian cells in culture, or all cultured vertebrate cells that produce EPO." (emphasis in original). While this broad entitlement question may be important as a policy matter, where, as here, we have applicable precedents, we are bound by the specific inquiries they mandate. Here, we, as did the district court, look to the requirements of § 112 as interpreted by our precedent. In short, the district court cannot have committed legal error by faithfully following controlling precedent of this court.

Lastly, the dissent emphasizes that omissions in the claim limitations and in the disclosures of the specifications "raised enablement issues." If the claims were still in prosecution before the PTO, [**64] perhaps the examiner could make an issue of such omissions. The dissent talks of what is "essential for the patentability of the claims." (emphasis added). But the question here is not patentability of application claims, but validity of issued claims that are presumed valid by statute. Now a heavy burden falls on the challenger. The district court found that the challenger had not carried that burden. It admitted that the questions were close -- indeed, it found invalidity proven, but only by a preponderance. Hence, rather than refusing to decide questions of validity under § 112, it did decide them under the proper standard of proof. We see no reversible error.

III

Having addressed the claim interpretation and § 112 issues, we move to [HN17] the second step of the infringement analysis: comparison of the properly construed claims to the accused product or process. See, e.g., *CCS Fitness, 288 F.3d at 1365, 62 USPQ2d at 1662.* Our review of this step differs depending upon whether the issue of infringement was resolved on summary judgment or after a full trial. See *Cole v. Kimberly-Clark Corp., 102 F.3d 524, 528, 41 USPQ2d 1001, 1004 (Fed. Cir. 1996).* [**65] In the case of summary judgment, as with claim 1 of the *'422* patent, we review de novo the trial court's finding that there was no genuine issue as to any material fact regarding infringement. *Id., 41 USPQ2d at 1004; Fed. R. Civ. P. 56(c).* After a full bench trial, infringement is a question of fact that we review, of course, for clear error. *Ultra-Tex Surfaces, Inc. v. Hill Bros. Chem. Co., 204 F.3d 1360, 1363, 53 USPQ2d 1892, 1895 (Fed. Cir. 2000).* When JMOL is entered under *Fed. R. Civ. P. 52(c)*, as with the *'698* and *'080* patents, we review the district court's determination for clear error, as if it had been entered at the close of all the evidence. *Yamanouchi Pharm. Co. v. Danbury Pharmacal, Inc., 231 F.3d 1339, 1343, 56 USPQ2d 1641, 1643 (Fed. Cir. 2000).* Anchored in the proper scope of review for each claim in dispute, we now address the trial court's infringement analysis.

[*1340] A. The *'933* Patent

Amgen asserted the following three claims of the *'933* patent against TKT:

> 1. A non-naturally occurring erythropoietin glycoprotein product having the in vivo biological activity of causing bone marrow cells [**66] to increase production of reticulocytes and red blood cells and having glycosylation which differs from that of human urinary erythropoietin.
>
> 2. The non-naturally occurring EPO glycoprotein product according to claim 1 wherein said product has a higher molecular weight than human urinary EPO as measured by SDS-PAGE.
>
> 9. A pharmaceutical composition comprising an effective amount of a glycoprotein product effective for erythropoietin therapy according to claim 1, 2, 3, 4, 5, or 6 and a pharmaceutically acceptable diluent, adjuvant or carrier.

Critical for our purposes is the final limitation of claim 1, which states that the claimed glycoprotein has "glycosylation which differs from that of human urinary erythropoietin." Glycosylation is the addition of carbohydrate side chains to amino acid residues in protein sequences to form glycoproteins. Encyclopedia of Molecular Biology at 1047. At the Markman hearing, Amgen asserted that the phrase meant "the attached carbohydrate groups differ when analyzed by standard prior art techniques known as of 1983-84." TKT argued, by contrast, that it meant "the carbohydrate groups attached to side chains of the erythropoietin [**67] polypeptide backbone differ by Western blot analysis and SDS/PAGE and carbohydrate composition analysis from those of human urinary erythropoietin to at least the degree described in the patents-in-suit."

Thus, the primary difference concerned which, if any, techniques were acceptable to determine whether the glycosylation was different. The district court found that the examples in the specification teach three

measurement methods, but that they failed to limit "glycosylation which differs" to those methods. The court ruled, therefore, that the phrase means: "Glycosylation as to which there is a detectable difference based upon what was known in 1983-1984 from that of human urinary erythropoietin, having in mind that the patent holder, Amgen, taught the use of this Western blot, SDS-PAGE and monosaccharide test." *Amgen, 126 F. Supp. 2d at 91-92, 57 USPQ2d at 1463.*

It is undisputed that in 1983, there were at least two analytical techniques available for detecting differences in glycosylation between two glycoproteins. SDS-PAGE is a type of gel electrophoresis in which the glycoprotein of interest is bound to a charged compound that denatures the glycoprotein, [**68] which in turn is subjected to an electric field; glycoproteins of different molecular weight (reflecting their different glycosylations) will migrate through the electric field at different speeds. *Id. at 124, 57 USPQ2d at 1488.* Isoelectric focusing ("IEF"), a second technique known to artisans in 1983, is similar to SDS-PAGE except that it determines the pH at which a protein is electrically neutral because the charge is placed in the gel in the form of a pH gradient, rather than on the glycoprotein itself. *Id. at 125, 57 USPQ2d at 1488.* The data obtained by both these methods can be visualized by Western blot, allowing an approximation of the molecular weight.

There was little dispute that any of these tests could be used to determine the glycosylation of a glycoprotein. Indeed, the district court noted that the testimony of an Amgen witness, Dr. Cummings, "would discharge Amgen's duty of showing by a preponderance of the evidence that HMR4396 has glycosylation which differs from that of human urinary EPO." *Id. at 127, 57 USPQ2d at 1490.* However, the [*1341] court also credited evidence that indicated two uEPO preparations produced from [**69] the same batch of starting materials could nevertheless have different glycosylation patterns. *Id. at 129, 57 USPQ2d at 1492* ("[A] skilled artisan in 1984 would have understood that urinary erythropoietin samples obtained using different purification methods could have different glycosylation. As a result, the glycosylation of human urinary erythropoietin was in 1984, and continues to be, a moving target."). Consequently, because the district court concluded that the patent failed to identify a single standard by which the "difference" could be measured, it held that TKT did not infringe and the *'933* patent was invalid for failure to satisfy *35 U.S.C. § 112:*

> The claim language of the *'933* patent, however, presupposes that the glycosylation of urinary erythropoietin is a fixed, identifiable marker against which the glycosylation of recombinant EPOs can be measured. Yet, how can one prove that a recombinant EPO has glycosylation which differs from that of urinary EPO when the glycosylation of urinary EPO itself varies? The Court need not answer this conundrum. All that need be said is that Amgen's showing that GA-EPO has glycosylation [**70] which differs from but one of the many heterogeneous urinary EPOs is insufficient to carry its burden of proving infringement by a preponderance of the evidence that TKT infringes the claim limitation.

Id. at 129, 57 USPQ2d at 1492.

Amgen argues on appeal that an ordinarily skilled artisan in 1984 would have understood, based upon the patent disclosure, that there were two principal processes for purifying uEPO, with the technique taught by Miyake (SDS-PAGE) recognized as the standard. It asserts that it carried its burden of proving infringement because its empirical evidence "unequivocally demonstrated the glycosylation difference between Miyake-purified uEPO and the accused product." But it seems to us that Amgen has failed to address the trenchant question on this issue, i.e., whether uEPO is necessarily glycosylated in the same way. Amgen deals rather cavalierly with the question in both its principal and reply brief, stating summarily that the district court erred and suggesting that the question is unimportant.

By definition, one must know what the glycosylation of uEPO is with certainty before one can determine whether the claimed glycoprotein [**71] has a glycosylation different from that of uEPO. In its discussion characterizing recombinant glycoprotein products, the specification of the *'933* patent does not direct those of ordinary skill in the art to a standard by which the appropriate comparison can be made. See *'933* patent, col. 28, line 33 - col. 29, line 7. The district court considered evidence that experiments conducted by Amgen in 1984 showed that different urinary EPO preparations had different glycosylation. For example, EPO purified from the urine of a single patient ("Lot 82") using a modified Miyake procedure was shown to have a different glycosylation from other human uEPO (taken from Goldwasser). *Amgen, 126 F. Supp. 2d at 129, 57 USPQ2d at 1491-92.* And so, even assuming that Amgen is correct that one of ordinary skill in the art would have understood the benchmark test for glycosylation to be Miyake, its contention still fails. As the district court noted, the Miyake article provides a method of

purification, but hardly suggests uniformity of glycosylation of the human uEPO studied:

> The 1977 Miyake et al. publication, for example, describes the purification from the same starting material [**72] of two homogeneous urinary EPO preparations (Fraction II and Fraction IIIA) that had about the same potency in terms of biological activity. Fractions II and IIIA [*1342] . . . had different carbohydrate compositions and, therefore, differed from each other in glycosylation. Thus, these two uEPO preparations, though produced by the same procedure (*Miyake) and derived from the same batch of starting material, nonetheless had different glycosylation.

Id. at 129, 57 USPQ2d at 1491; see also Miyake, Purification of Human Erythropoietin, J. Bio. Chem. 5558, 5562 (1977) ("In spite of our finding of similar potency and molecular size, these two preparations [Fractions II and IIIA] must be considered different. The chemical basis for this difference is now being studied."). Amgen fails to controvert or otherwise address this evidence in its cross-appeal.

[HN18] Under *35 U.S.C. § 112* P 2, a patent applicant is required, at the close of his specification, to "particularly point[] out and distinctly claim[] the subject matter the applicant regards as his invention." The requirement of claim definiteness set out in § *112 P 2* assures [**73] that claims in a patent are "sufficiently precise to permit a potential competitor to determine whether or not he is infringing." *Morton Int'l, Inc. v. Cardinal Chem. Co., 5 F.3d 1464, 1470, 28 USPQ2d 1190, 1195 (Fed. Cir. 1993).* The standard of indefiniteness is somewhat high; a claim is not indefinite merely because its scope is not ascertainable from the face of the claims. Cf., e.g., *LNP Eng'g Plastics, Inc. v. Miller Waste Mills, Inc., 275 F.3d 1347, 1359-60, 61 USPQ2d 1193, 1202 (Fed. Cir. 2001)* (affirming district court finding that patent was not indefinite, despite testimony from a co-inventor that he did not understand what the claim limitation "substantially completely wetted" meant). Rather, a claim is indefinite under § *112 P 2* if it is "insolubly ambiguous, and no narrowing construction can properly be adopted." *Exxon Research & Eng'g Co. v. United States, 265 F.3d 1371, 1375, 60 USPQ2d 1272, 1276 (Fed. Cir. 2001); Allen Eng'g Corp. v. Bartell Indus., Inc., 299 F.3d 1336, 1349, 63 USPQ2d 1769, 1776 (Fed. Cir. 2002)* ("It is not our function to rewrite [indefinite] claims to preserve their [**74] validity."). Applying these legal maxims to the facts of this case, we agree with the district court that the claims requiring "glycosylation which differs" are invalid for indefiniteness.

We find erroneous, however, its conclusion that invalidity for indefiniteness should be found only in the alternative. [HN19] A claim is indefinite if, when read in light of the specification, it does not reasonably apprise those skilled in the art of the scope of the invention. See *Solomon v. Kimberly-Clark Corp., 216 F.3d 1372, 1378, 55 U.S.P.Q.2D (BNA) 1279, 1282 (Fed. Cir. 2000)* (citing *Personalized Media Comm., LLC v. ITC, 161 F.3d 696, 705, 48 USPQ2d 1880, 1888 (Fed. Cir. 1998)).* So it is here. Recognizing that it was faced with a "conundrum" regarding claim construction, the court held that the patent was not infringed because Amgen could not meet its burden simply by showing "that GA-EPO has glycosylation which differs from but one of the many heterogeneous urinary EPOs." *Amgen, 126 F. Supp. 2d at 129, 57 USPQ2d at 1492.* That the court recognized that one of ordinary skill in the art would have been faced with this "conundrum" should have ended [**75] the inquiry, for such ambiguity in claim scope is at the heart of the definiteness requirement of *35 U.S.C. § 112* P 2. One cannot logically determine whether an accused product comes within the bounds of a claim of unascertainable scope. Accordingly, the finding that TKT does not infringe the *'933* patent is vacated and the finding that the *'933* patent is invalid under § 112 is affirmed.

B. The *'080* Patent

Claims 2-4 of the *'080* patent are at issue:

> 2. An isolated erythropoietin glycoprotein having the in vivo biological activity [*1343] of causing bone marrow cells to increase production of reticulocytes and red blood cells, wherein said erythropoietin glycoprotein comprises the mature erythropoietin amino acid sequence of FIG. 6 and is not isolated from human urine.
>
> 3. A non-naturally occurring erythropoietin glycoprotein having the in vivo biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells, wherein said erythropoietin glycoprotein comprises the mature erythropoietin amino acid sequence of FIG. 6.

> 4. A pharmaceutical composition comprising a therapeutically effective amount of an [**76] erythropoietin glycoprotein product according to claim 1, 2, or 3.

The critical limitation of the asserted claims in the *'080* patent is the requirement that the erythropoietin glycoprotein "comprise[] the mature erythropoietin amino acid sequence of Fig. 6." The court construed the claim term "mature erythropoietin amino acid sequence of Figure 6" that appears in claims 4 and 6 of the *'698* patent and claims 2 and 4 of the *'080* patent. The dispute here arises out of a mistake in the specification. At the time the patent was drafted, it was believed that the sequence included 166 amino acids, and this belief is depicted in Figure 6. In fact, later research demonstrated that the full sequence was actually 165 amino acids; the last (arginine) is actually cleaved off prior to the protein's secretion from the cell. Amgen argued that the reference to Figure 6 was irrelevant, even if the figure had too many amino acids, because it still showed the "mature [i.e., 165] erythropoietin amino acid sequence." TKT argued that the reference to Figure 6 required the term to be construed as depicted in Figure 6, and thus with 166 amino acids. Following trial, n12 the court adopted TKT's [**77] proposal, relying on what it considered key language in the specification supporting that construction: "Fig. 6 thus serves to identify the primary structural conformation (amino acid) sequence of mature human EPO as including 166 specified amino acid residues . . . ." *'080* patent, col. 12, lines 3-5. *Amgen, 126 F. Supp. 2d at 86-87, 57 USPQ2d at 1459.*

> n12 The court declined to rule on this issue at the Markman hearing, instead choosing to take the matter under advisement. See *126 F. Supp. 2d at 87, 57 USPQ2d at 1459.*

In total, Figure 6 consists of five separate figures denominated Figs. 6A through 6E, which collectively disclose the sequence of human genomic EPO DNA and the encoded EPO. The detailed description in the *'080* patent indicates that the specificity of Figure 6 is not to be lightly disregarded:

> Fig. 6 thus serves to identify the primary structural conformation (amino acid sequences) of mature human EPO as including 166 specified amino acid residues (estimate [**78] M.W.=18,399). Also revealed in the Figure is the DNA sequence coding for a 27 residue leader sequence along with 5' and 3' DNA sequences which may be significant to promoter/operator functions of the human gene operon. Sites for potential glycosylation of the mature human EPO polypeptide are designated in the Figure by asterisks. It is worthy of note that the specific amino acid sequence of Fig. 6 likely constitutes that of a naturally occurring allelic form of human erythropoietin. Support for this position is found in the results of continued efforts at sequencing of urinary isolates of human erythropoietin which provided the finding that a significant number of erythropoietin molecules therein have a methionine at residue 126 as opposed to a serine as shown in the Figure.

'080 patent, col. 21, lines 29-40.

When the district court revisited the "Figure 6" issue, it concluded that the [*1344] language of the claims, read in conjunction with the portion of the specification excerpted above, clearly identified the mature erythropoietin amino acid sequence as exactly depicted in Figure 6. In so doing, the court expressly rejected Amgen's contention that the claim should be read as covering [**79] the mature amino acid sequence, of erythropoietin, whatever its number of amino acids. *Amgen, 126 F. Supp. 2d at 100, 57 USPQ2d at 1470* ("Had Amgen claimed only 'the mature erythropoietin amino acid sequence' without associating or linking that amino acid sequence to Figure 6 its argument that its claims cover whatever sequence (whether it contained 165 or 166 amino acids) is ultimately secreted by the cell might have more momentum."). The district court therefore found at the close of Amgen's case that HMR4396 does not literally infringe the asserted claims of the *'080* patent.

The issue of infringement under the doctrine of equivalents was much closer, and likewise centered on the "Figure 6" limitation. n13 The district court concluded that Amgen had proven by a preponderance of the evidence that the 165 amino acid sequence satisfied the function-way-result test, crediting in particular the testimony of Dr. Lodish that TKT's missing arginine residue (the 166th amino acid appearing in Figure 6) does not affect the in vivo biological activity of its EPO product. *Id. at 133, 57 USPQ2d at 1495.* In reaching its conclusion, the court rejected TKT's [**80] argument that Amgen was not entitled to any range of equivalents under *Festo Corp. v. Shoketsu Kinzoku Kogyo Kabushiki, 234 F.3d 558, 566, 56 USPQ2d 1865, 1870 (Fed. Cir.*

2000), because during prosecution it had narrowed the scope of the claim for reasons related to patentability. The parties have cross-appealed on this patent, with Amgen asserting that the district court erred by finding no literal infringement and TKT continuing to press its estoppel theory as a basis for denying any range of equivalents.

n13 The district court held that every other limitation of the asserted claims in the *'698* patent were met literally by the accused product/process. *Amgen, 126 F. Supp. 2d at 132-33, 57 USPQ2d at 1493.* Thus, whether equivalent infringement occurred turned on whether the "Figure 6" limitation was equivalently met.

Naturally, Amgen continues to focus on the "mature" portion of the relevant claim limitations to support its argument that the trial court erred by finding no literal [**81] infringement. According to Amgen, the practical result of the trial court's conclusion is to read out from the claims the preferred embodiment of the invention because the specification makes clear that "mature" human EPO is that form which circulates in the blood, i.e., the 165 amino acid form that has already been secreted. This argument strains reason to its breaking point; our reading of the patent, like the district court's, will support no such interpretation.

Amgen's argument is based upon a misconstruction of the term "including" that evinces a misunderstanding of the plain meaning of that term, as well as the term "comprise," which appears in the *'080* patent claims. n14 [HN20] "Comprising is a term of [*1345] art used in claim language which means that the named elements are essential, but other elements may be added and still form a construct within the scope of the claim." *Genentech, Inc. v. Chiron Corp., 112 F.3d 495, 501, 42 USPQ2d 1608, 1633 (Fed. Cir. 1997).* The word "include" means the same thing. See *Hewlett-Packard Co. v. Repeat-O-Type Stencil Mfg. Corp., Inc., 123 F.3d 1445, 1451, 43 USPQ2d 1650, 1655 (Fed. Cir. 1997)* ("The claim term 'including' [**82] is synonymous with 'comprising,' thereby permitting the inclusion of unnamed components."); see also Webster's II New Riverside University Dictionary 619 (1984) ("include: 1. To have or take in as a part or member: CONTAIN; 2. To put into a group class, or total."). Thus, a claim reciting "a widget comprising A and B," for example, would be infringed by any widget containing A and B, no matter that C, D, or E might be present.

n14 Amgen argues: "The specification describes the mature amino acid sequence of human EPO as 'including' -- not 'limited to' -- the 1-166 sequence. Properly construed, Lin's claimed sequence -- the mature sequence -- includes the fully processed form of any glycoprotein having the Figure 6 sequence. That includes both the 1-165 and the 1-166 amino acid sequences of Figure 6. Only this construction affords 'mature' its proper meaning, and includes Lin's preferred embodiment."

If, then, as the specification states, "the primary structural conformation (amino acid sequence) of mature [**83] human EPO as including 166 specified amino acid residues," it is simply illogical for Amgen to argue that that means anything other than, at minimum, the 166 amino acids shown in Figure 6. This is verified by the fact that *'080* claims 2 and 3 claim an erythropoietin glycoprotein "comprising the mature erythropoietin amino acid sequence of Fig. 6 . . . ." Again, read properly in light of the term "comprising," this means that the claimed glycoprotein must have -- at minimum -- all 166 amino acids shown in Figure 6.

Turning to the finding of infringement under the doctrine of equivalents, TKT asserts that Amgen should be estopped from obtaining such coverage under Festo. Specifically, TKT alleges that the "mature amino acid sequence of Figure 6" limitation that appears in the *'080* patent was added to overcome a double-patenting rejection, and therefore constitutes an amendment related to patentability. We agree.

The district court correctly found that the amendment, although voluntary, was made to avoid a "same invention" double patenting rejection, *Amgen, 126 F. Supp. 2d at 135, 57 USPQ2d at 1496,* and although the Supreme Court reversed our decision in [**84] Festo and rejected the notion of an absolute bar to the doctrine of equivalents, it agreed with our holding "that [HN21] a narrowing amendment to satisfy any requirement of the Patent Act may give rise to an estoppel." *Festo Corp. v. Shoketsu Kinzoku Kogyo Kabushiki, 535 U.S. 722, 122 S. Ct. 1831, 1839, 152 L. Ed. 2d 944 (2002).* Contrary to the district court's conclusion, [HN22] "'same invention' double patenting is based upon *35 U.S.C. § 101,* which states that an inventor may obtain 'a patent' for an invention." *In re Lonardo, 119 F.3d 960, 965, 43 USPQ2d 1262, 1266 (Fed. Cir. 1997)* (emphasis added). Therefore, the district court's finding of equivalent infringement of the *'080* patent is vacated and remanded for an analysis under the narrow ways of rebutting the Supreme Court's presumption of estoppel. *Festo, 122 S. Ct. at 1839.*

C. The *'698* Patent

The *'698* patent is directed generally to a process for producing a glycosylated erythropoietin polypeptide. Claims 4-9 are at issue. Independent claims 4 and 6 read as follows:

> 4. A process for the production of a glycosylated erythropoietin polypeptide [**85] having the in vivo biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells comprising the steps:
>
> > a) growing, under suitable nutrient conditions, vertebrate cells comprising promoter DNA, other than human erythropoietin promoter DNA, operatively [*1346] linked to DNA encoding the mature erythropoietin amino acid sequence of FIG. 6; and
> >
> > b) isolating said glycosylated erythropoietin polypeptide expressed by said cells
>
> 6. A process for the production of a glycosylated erythropoietin polypeptide having the in vivo biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells comprising the steps of:
>
> > a) growing, under suitable nutrient conditions, vertebrate cells comprising amplified DNA encoding the mature erythropoietin amino acid sequence of FIG. 6; and
> >
> > b) isolating said glycosylated erythropoietin polypeptide expressed by said cells.

Infringement of dependent claims 5 and 7-9 rises or falls with the analysis that applies to independent claims 4 and 6. n15 The phrase "operatively linked" appears in claim 4 of the *'698* patent, and is related by dependency [**86] to claims 5 and 9. According to the district court, the phrase relates to the relationship between promoter DNA and the DNA that is transcribed downstream from the promoter DNA. Amgen contended that the phrase means "positioned such that it provides for initiation of transcription of a gene." TKT argued that the term means positioned adjacent "to the DNA encoding EPO in a way that maintains the capability to initiate transcription of EPO DNA." In other words, Amgen argued that the words "operatively linked" imposed no spatial restriction, whereas TKT contended that because the patent allegedly taught placing the promoter DNA immediately adjacent to the DNA encoding EPO, the term "operatively linked" ought be limited by location. The district court held that the term "operatively linked" means "the promoter DNA is linked to the EPO DNA is such a way that maintains the capability of the promoter DNA to initiate transcription of the EPO DNA." *Amgen, 126 F. Supp. 2d at 90, 57 USPQ2d at 1462.*

> n15 Claim 5 claims "the process of claim 4 wherein said promoter DNA is viral promoter DNA." Claim 7 claims "the process of claim 6 wherein said vertebrate cells further comprise amplified marker gene DNA." Claim 8 claims "the process of claim 7 wherein said amplified marker gene DNA is Dihydrofolate reductase (DHFR) gene DNA." And claim 9 claims "the process according to claims 2, 4 and 6 wherein said cells are mammalian cells."

[**87]

The district court granted TKT summary judgment of non-infringement of independent claims 4 and 6 (and hence dependent claims 5 and 7-9) of the *'698* patent because it found that Amgen had failed to carry its Rule 52(c) burden. *Id. at 102, 57 USPQ2d at 1471.* Amgen assails this conclusion as not in accordance with law, inasmuch as the differences considered dispositive by the district court are not claimed and thus have no bearing on a proper infringement analysis. In fact, according to Amgen, the district court neglected to identify any limitation of the *'698* patent that the accused process fails to literally meet, and also failed to explain why, in the absence of literal infringement, those limitations were not otherwise equivalently met. We agree with Amgen, and therefore conclude vacatur is appropriate.

The district court properly recognized that the infringement analysis of process claims is necessarily different from that for product claims. See *id. at 102, 57 USPQ2d at 1471* ("The process patent gives notice to competitors that the steps described therein are not to be repeated to achieve the same result. Thus, whereas in the

product patent [**88] context, differences in process are meaningless, here, in the process [*1347] patent context, these differences mean everything."). But after a correct discussion of the differences in the infringement analysis, the court eschewed the cardinal principle that the accused device must be compared to the claims rather than to a preferred or commercial embodiment. Id. ("Based on . . . the many differences between Amgen's and TKT's processes . . . Amgen's proof of infringement on the *'698* patent [is] insufficient . . . .") (emphasis added).

For example, the court concluded that a fundamental distinction between the respective processes was that TKT employs homologous rather than heterologous recombination, whereas "in order to make EPOGEN (R), Amgen transfects [CHO] cells with a vector that contains both viral promoter DNA and the human EPO gene." Id. This clear reference to the preferred embodiment of Example 10, which the district court considered "the process most heavily relied upon by Amgen in its patent," *id. at 103, 57 USPQ2d at 1472,* misses the point that none of the claims at issue contain such a limitation. And apart from the limitations of the asserted [**89] claims, the differences in the two processes are wholly irrelevant to the infringement analysis.

The district court likewise found material the fact that TKT places its promoter and enhancer farther upstream than does Amgen. In light of the court's claim construction, however, it would seem TKT satisfies the "operatively linked" limitation, as there is no question that TKT's promoter causes its intended functional effect. In any event, the trial court once again compared the accused process by reference to an example rather than the claimed process:

> As explained in Example 7 and illustrated in Figure 4, Amgen created the vector by cleaving, with BstEII restriction endonucelases . . . 'at a position which is 44 base pairs 5' to the initiating ATG coding for the pre-peptide and approximately 680 base pairs 3' to the HindIII restriction site' . . . . TKT's process has within the DNA sequence upstream of the codons that express the EPO polypeptide several ATG sites . . . . The court finds that such a process is sufficiently different from that encompassed by Amgen's invention that judgment of non-infringement should follow.

Id.

Again, this was legal error insofar [**90] as the infringement analysis is not tied to the asserted claims. We therefore vacate and remand so that the court may conduct a proper infringement inquiry in the first instance, comparing the accused device to the properly construed claims without limiting their scope to the examples in the specification or other limitations that are not properly a part of claims 4-9.

D. The *'422* Patent

Claim 1 of the *'422* patent, the only one in dispute, claims "[a] pharmaceutical composition comprising a therapeutically effective amount of human erythropoietin and a pharmaceutically acceptable diluent, adjuvant or carrier, wherein said erythropoietin is purified from mammalian cells grown in culture." In the Markman hearing, Amgen contended the phrase "purified from mammalian cells grown in culture" meant "purified from the in vitro culture in which the mammalian cells have been grown," whereas TKT argued that it meant "obtained in a substantially homogeneous state from the mammalian cells in which it was produced and not from the cell culture media." Concluding that TKT's construction would exclude the patent's preferred embodiment (Example 10), the court read the phrase "mammalian [**91] cells grown in culture" as a whole to encompass purification techniques from the [*1348] cells or the cell culture medium. *Id. at 88-89, 57 USPQ2d at 1460-61.* As indicated earlier, the district court immediately turned to and granted Amgen's motion for summary judgment of infringement of the *'422* patent at the close of the Markman hearing.

According to the district court, it was clear from the beginning that the accused product met most limitations of claim 1. That HMR4396 was a pharmaceutical composition that contained a therapeutically effective amount of human erythropoietin was plain, in view of the Investigational New Drug Application ("INDA") that TKT filed with the Food and Drug Administration. *Id. at 94-95, 57 USPQ2d at 1465.* The district court further concluded that HRM4396 contained "a pharmaceutically acceptable diluent, adjuvant or carrier" in view of the testimony of TKT's Rule 30(b)(6) designee, who testified that the HRM4396 recovered in bulk from the culturing of human cells was diluted with a phosphate buffer to control the pH and provide a product of desired strength. See *id. at 95, 57 USPQ2d at 1466.* The sole remaining [**92] issue, then, was whether the accused product was "purified from mammalian cells grown in culture." Rather than taking the utterly untenable position that humans are not mammals, TKT conceded infringement under the court's claim construction. *Id. at 95, 57 USPQ2d at 1466.*

TKT tries three different tactics on appeal to escape this concession of infringement. First, TKT argues that "mammalian cells," as the phrase is used in the *'422* patent, do not include its cells because Amgen excluded the use of human cells to produce human EPO from its invention. Second, TKT asserts that the finding of infringement was in error because the patent specification defines pharmaceutical compositions "as comprising 'polypeptides of the invention'," and HRM4396 is not a "polypeptide of the invention" inasmuch as the invention is "uniquely characterized" by (and hence limited to) exogenous EPO DNA. Finally, TKT challenges the finding of infringement because, it asserts, the intrinsic evidence limits the phrase "purified from mammalian cells grown in culture" to purification that takes place inside the cells, and not -- like TKT -- from the culture media. n16 As infringement of the *'422* [**93] patent was granted on summary judgment, we review the district court's conclusion de novo, applying the same standard applied by the trial court. *Schering Corp. v. Amgen, Inc., 222 F.3d 1347, 1351, 55 USPQ2d 1650, 1653 (Fed. Cir. 2000).* Under this standard, we agree with the trial court that a grant of summary judgment of infringement of the *'422* patent was warranted.

n16 The basis for this argument is that claim 2 of the *'698* patent recites recombinant EPO "isolated from the host cell or the medium of its growth." Therefore, asserts TKT, "Amgen also knew how to claim what it now seeks, but failed to do so."

We cannot accept, for the reasons already stated, TKT's proposed reading of the claim term "mammalian" and its attempt to import the term exogenous into the claims; we therefore reject out of hand the contention that Amgen expressly excluded the use of human cells to express EPO and the use of endogenous DNA from the scope of its invention. Thus, the issue resolves to a narrow one: the [**94] accused product, HRM4396, infringes *'422* patent claim 1 unless TKT is correct that the claim limitation "purified from mammalian cells grown in culture" means that the EPO product must be recovered directly from the cell, and not from the culture medium.

At the Markman hearing, Amgen contended the phrase means "purified from the in vitro culture in which the mammalian [*1349] cells have been grown"; TKT argued that it means "obtained in a substantially homogeneous state from the mammalian cells in which it was produced and not from the cell culture media. The trial court read the phrase to encompass purification techniques from the cells or the cell culture medium because to do otherwise, it found, would exclude the patent's preferred embodiment as disclosed in Example 10. *Amgen, 126 F. Supp. 2d at 88-89, 57 USPQ2d at 1461.*

Example 10 "describes expression systems employing Chinese hamster ovary (CHO) DHFR-cells and the selectable marker, DHFR." *'422* patent, col. 25, lines 38-40. As a part of the description, the example discloses that gene amplification in cell culture media is possible to increase productivity of the targeted recombinant EPO product. After describing [**95] an example of such a gene amplification system, the specification goes on to state: "The productivity of the EPO producing CHO cell lines described above can be improved by appropriate cell culture techniques. The propagation of mammalian cells in culture generally requires the presence of serum in the growth media. A method for production of erythropoietin from CHO cells in media that does not contain serum greatly facilitates the purification of erythropoietin from the culture media." Id., col. 27, lines 8-14 (emphasis added). We agree with the district court that this disclosure -- the undisputed preferred embodiment of the invention -- contemplates purification of erythropoietin from the culture media. See also *'933* patent, col. 28, lines 28-32 ("Mammalian cell expression products may be readily recovered in substantially purified form from culture media using HPLC (C4) employing an ethanol gradient, preferably at pH7." (emphasis added)).

TKT does not challenge the district court's conclusion regarding the disclosure of Example 10. Accordingly, TKT's challenge ultimately must fail unless we read the preferred embodiment out of the claims, but rare is the case where [**96] we should or will do so. [HN23] A claim interpretation that reads out a preferred embodiment "is rarely, if ever, correct and would require highly persuasive evidentiary support." *Vitronics Corp. v. Conceptronic, Inc., 90 F.3d 1576, 1583, 39 USPQ2d 1573, 1578 (Fed. Cir. 1996).* We have done so only one time -- in an instance where the patent applicant limited the full scope of the claim language to omit the preferred (and only disclosed) embodiment in order to overcome an examiner's rejection. See *Elekta Instr. S.A. v. O.U.R. Scientific Int'l, Inc., 214 F.3d 1302, 1308, 54 USPQ2d 1910, 1914 (Fed. Cir. 2000).* The present case lacks the "persuasive evidentiary support" necessary for us to read the claims so as to exclude the preferred embodiment disclosed in Example 10; we therefore decline to do so.

E. The *'349* Patent

The *'349* patent contains one method claim and six product claims that are drawn generally to types of

vertebrate cells grown in culture. At issue are claims 1, 3-4, and 6-7:

> 1. Vertebrate cells which can be propagated in vitro and which are capable upon growth in culture of producing erythropoietin in the medium of their growth [**97] in excess of 100 U of erythropoietin per 106 cells in 48 hours as determined by radioimmunoassay, said cells comprising non-human DNA sequences that control transcription of DNA encoding human erythropoietin.
>
> 3. Vertebrate cells according to claim 1 capable of producing in excess of 1000 U erythropoietin per 106 cells in 48 hours.
>
> 4. Vertebrate cells which can be propagated in vitro which comprise transcription [*1350] control DNA sequences, other than human erythropoietin transcription control sequences, for production of human erythropoietin, and which upon growth in culture are capable of producing in the medium of their growth in excess of 100 U of erythropoietin per 106 cells in 48 hours as determined by radioimmunoassay.
>
> 6. Vertebrate cells according to claim 4 capable of producing in excess of 1000 U erythropoietin per 106 cells in 48 hours.
>
> 7. A process for producing erythropoietin comprising the step of culturing, under suitable nutrient conditions, vertebrate cells according to claim 1, 2, 3, 4, 5, or 6.

Each of the claims contain the limitation "non-human DNA sequences that control transcription" that appears in claim 1 of the *'349* patent or the limitation [**98] "transcriptional control DNA sequences, other than human erythropoietin transcription control sequences" that appears in claim 4 of the *'349* patent. Transcription is the process whereby RNA polymerase copies genetic information contained in a DNA nucleotide sequence into an RNA sequence. It is a critical step in the expression of proteins like erythropoietin and is itself controlled by specific DNA sequences. According to the patent, "transcription control sequences" is the collective term for DNA sequences that not only "provide a site for initiation of transcription into mRNA," but also are capable of binding proteins that determine "the frequency (or rate) of transcriptional initiation." *'349* patent, col. 2, lines 3-12.

Amgen contended that this phrase means "non-human DNA sequences that are able to initiate or regulate RNA synthesis from EPO DNA." TKT argued that the phrase means "DNA sequences which did not originate in the human genome, which initiate and regulate RNA synthesis of adjacent DNA, and which replace the human EPO transcription control sequences." By including the term "adjacent DNA" in its construction, TKT sought to require the DNA sequences that control transcription [**99] to be located in a position adjacent to the gene segment intended to be expressed. Furthermore, TKT contended that in order to "control" transcription, the DNA sequences must both initiate and regulate the transcription of a gene. Amgen objected to the use of "and," preferring a construction that required DNA sequences either to initiate or regulate transcription. Finally, the parties disagreed as to the meaning of "non-human." Amgen argued that "non-human" means "not part of the human genome," whereas TKT contended it meant "not originating in the human genome." n17

> n17 The importance of this distinction is that, because it is scientifically arguable that viral DNA originates in the human genome, the viral promoter DNA that TKT employs thus might not fall within the meaning of the claim.

First, the court rejected TKT's position and concluded that "non-human" DNA sequences are DNA sequences that are "not part of the human genome." The court similarly rejected TKT's "adjacent" language because "no claim term could [**100] reasonably be construed to be limiting the transcription control DNA sequences by their location." Finally, the court held that DNA sequences that control transcription are DNA sequences that initiate and regulate the processes of transcription. *Amgen, 126 F. Supp. 2d at 88, 57 USPQ2d at 1459-60.*

The district court entered judgment of noninfringement for TKT on method claim 7 of the *'349* patent under an identical rationale to that used to grant judgment of noninfringement for the method claims of the *'698* patent. *Id. at 122, 57 USPQ2d at 1486.* As we have found the [*1351] court's analysis with respect to the *'698* patent to be legally unsupportable, see supra at 41-42, we likewise vacate the district court's judgment with respect to claim 7 of the *'349* patent and remand for further consideration. As to the product claims of the *'349* patent, the court held that each of claims 1, 3, 4, and

6 were literally infringed, and further held (alternatively) that claims 3 and 6 were equivalently infringed. n18

n18 We note also that the trial court granted summary judgment of infringement of the product claims of the *'349* patent. It modified its summary judgment finding (but reached the same result) with respect to the "controlling transcription" limitation in light of extensive trial testimony. *Amgen, 126 F. Supp. 2d at 118, 57 USPQ2d at 1485.* Accordingly, unlike the other limitations in the *'349* patent, we review the court's conclusion with respect to "controlling transcription" for clear error, even though it comes to us from a grant of summary judgment of infringement. Because TKT has not demonstrated clear error in the trial court's conclusion, we affirm the finding of infringement.

[**101]

Aside from the challenge, already rejected, to the trial court's construction of the term "vertebrate cells," TKT mounts a weak challenge to these findings of infringement apparently under the reverse doctrine of equivalents. n19

n19 The sum total of TKT's challenge to the infringement finding, aside from the "vertebrate" issue, is as follows: "[TKT] also does not use the 'transcription control sequences' of the *'349* patent. As the court found, [TKT]'s transcription control sequences are not only structurally different from Amgen's sequences but also function in a different way. Because of those differences in structure and function, [TKT] does not infringe the 'transcription control sequences' limitation in the *'349* claims."

[HN24] Under the reverse doctrine of equivalents, an accused product or process that falls within the literal words of a claim nevertheless may not infringe if the product or process "is so far changed in principle from a patented article that it performs the same or a similar function in [**102] a substantially different way." *Graver Tank & Mfg. Co. v. Linde Air Prod. Co., 339 U.S. 605, 608-09, 85 USPQ 328, 330, 94 L. Ed. 1097, 70 S. Ct. 854 (1950);* see generally Donald S. Chisum, 5A *CHISUM ON PATENTS § 18.04* (1999). This doctrine is equitably applied based upon underlying questions of fact, see *Scripps Clinic & Research Foundation v. Genentech, Inc., 927 F.2d 1565, 1581, 18 USPQ2d 1001, 1013 (Fed. Cir. 1991),* when the accused infringer proves that, despite the asserted claims literally reading on the accused device, "it has been so changed that it is no longer the same invention." *Del Mar Avionics, Inc. v. Quinton Instr. Co., 836 F.2d 1320, 1325, 5 USPQ2d 1255, 1259 (Fed. Cir. 1987)* (citing *Graver Tank, 339 U.S. at 608-09).*

We are not persuaded by TKT that this is a case where equity commands a determination of non-infringement despite its product literally falling within the scope of the asserted claims. TKT relies on findings of the district court regarding differences in the way the accused device controls transcription in the *'698* patent. It is true, as Amgen candidly admits, that the method by [**103] which TKT controls transcription is not identical. Whereas the patent describes placing the promoter DNA in close proximity, or even adjacent, to the EPO leader peptide, TKT places its promoter further upstream. But again, it is error to conduct infringement analyses in a vacuum, without reference to the claims at issue.

The vertebrate cells of the *'349* patent, as claimed, are comprised of non-human DNA sequences that control transcription of DNA encoding human erythropoietin. And "controlling transcription of DNA encoding human erythropoietin" simply [*1352] means initiating and regulating the process of transcription. *Amgen, 126 F. Supp. 2d at 88, 57 USPQ2d at 1460.* This limitation is met literally because the cytomegalovirus in TKT's R223 cells performs this function, *id. at 118, 57 USPQ2d at 1484,* notwithstanding TKT's reliance on the court's erroneous analysis of the *'698* patent method claims.

IV

Our affirmance of the district court's findings that certain of the asserted claims are infringed is not yet the coup de grce for TKT; non-frivolous validity issues remain. [HN25] One of the statutory requirements for patentability is that the invention for which [**104] a patent is sought was not known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention by the applicant. See *35 U.S.C. § 102*(a). Similarly, one is not entitled to a patent if the subject matter of the invention as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which the invention is directed. See id. § 103. TKT relies particularly on two items of prior art that allegedly render certain of the asserted claims anticipated under § 102(a) or obvious under § 103. We discuss each in turn.

A

TKT contends the asserted claims are anticipated by the work of Dr. Eugene Goldwasser ("Goldwasser"). Beginning in 1979-80, Goldwasser conducted a clinical study at the University of Chicago at Illinois in which he obtained a preparation of highly purified erythropoietin derived from human urine and administered approximately 10,000 units of human urinary EPO to three anemic patients. *Amgen, 126 F. Supp. 2d at 111, 57 USPQ2d at 1478.* Although this study showed an increase in reticulocyte count in all three [**105] patients, and an increase in erythroid cells, plasma iron clearance rate, and red cell mass in at least one patient, Goldwasser admitted that "there was no significant change in hematocrit in any patient." *Id. at 111-12, 57 USPQ2d at 1478.* And because there was no increase in hematocrit, Goldwasser testified in his deposition that he considered the study a failure. The district court concluded, as a result, that the study could not be invalidating anticipatory prior art: "Another's experiment, imperfect and never perfected will not serve either as an anticipation or as part of the prior art, for it has not served to enrich it." *Id. at 112, 57 USPQ2d at 1479* (quoting *Fromson v. Advance Offset Plate, Inc., 755 F.2d 1549, 1558, 225 USPQ 26, 33 (Fed. Cir. 1985)).*

The district court similarly concluded that Goldwasser did not render the patents obvious. Of paramount importance to the court was the fact that the prior art references, including Goldwasser, lacked Amgen's disclosure of the genetic sequence of EPO and failed to describe any transcription control sequences. Id. at 115, 57 USPQ2d at 1481. The court also considered the secondary [**106] factors -- particularly long-felt need and commercial success -- to be of high importance. Id. at 116, 57 USPQ2d at 1482 ("Before the advent of Amgen's product, whether EPO could actually produce a sustainable increase in a patient's hematocrit was not known. Furthermore, Amgen's EPO product, which was the first EPO-containing pharmaceutical composition to obtain FDA approval, has greatly improved the quality of life of chronic renal failure patients throughout the world. As a result, Dr. Lin received widespread public acclaim for his work.").

TKT assigns error to the district court's alleged blind acceptance of Goldwasser's assertion that the test was a failure without considering the contemporaneous [*1353] testimony of Goldwasser's collaborator, Dr. Baron, who reported to the Food and Drug Administration in 1984 that evidence of erythroid marrow stimulation was detected. In particular, according to TKT, the court erred by failing to "look[] at the definition of therapeutic effect in the specification." We agree that "therapeutically effective" must be defined in accordance with Markman v. Westview Instruments before this issue can be properly resolved, and we therefore vacate [**107] and remand for further proceedings with respect to Goldwasser.

For the Markman hearing in this case, ten terms were "pre-selected" based upon their relationship to Amgen's then-pending motion for summary judgment of infringement. Id. at 81, 57 USPQ2d at 1455. Whether those "pre-selected" terms were chosen by the court or the parties is unclear from the record, but what is clear is that "therapeutically effective" was not among them. And so the district court, assumedly viewing "therapeutically effective" as not in dispute, construed it in its discussion of the Goldwasser reference:

> Such evidence [of, e.g., increased erythroid marrow stimulation] should be outweighed by the fact that the actual production of mature red blood cells was not achieved and, as a result, hematocrit levels were unchanged. Because an increase in hematocrit and hemoglobin levels is the true mark of therapeutic effectiveness, Dr. Goldwasser's study, which revealed only inchoate indicators of red blood cell production, falls far short of anticipating claims requiring a therapeutic amount of human EPO.

Id. at 112, 57 USPQ2d at 1479 (second emphasis ours). Had "therapeutically [**108] effective" not been in dispute, no error would arise. [HN26] A district court may -- indeed, often must -- interpret or define a term in the claims that is not in dispute in order to provide a proper context for the discussion of the terms that are in dispute. See, e.g., *DeMarini Sports v. Worth, Inc., 239 F.3d 1314, 1323, 57 USPQ2d 1889, 1893-94 (Fed. Cir. 2001).* But here, the term "therapeutically effective" is in dispute because it is central to whether Goldwasser is properly considered prior art. See *In re Donohue, 766 F.2d 531, 226 USPQ 619 (Fed. Cir. 1985)* (holding that a non-enabled disclosure will not suffice as § 102 prior art).

Although the endgame in the treatment of chronically anemic patients is to increase the hematocrit, as recognized by the district court, the claim term "therapeutically effective" must be understood in light of the specification of which it is a part. And that specification appears to teach that results in addition to simply an increase in hematocrit can provide effective therapy. See *'933* patent, col. 33, lines 19-31 ("[The claimed polypeptide products] are conspicuously suitable for use in erythropoietin therapy [**109] procedures . . . to develop any or all of the effects heretofore attributed in vivo to EPO, e.g., stimulation of reticulocyte response

. . ., erythrocyte mass changes . . ., and, as indicated in Example 10, increasing hematocrit levels in mammals." (emphasis added)).

Amgen asserts that the district court's construction of "therapeutically effective" is supported by admissions of TKT's experts that the term means "increasing and maintaining the patient's hematocrit to normal or near normal levels." But [HN27] the relevant question is not whether one of ordinary skill would so understand the term, but whether that term should be limited based upon the express disclosure in the specification. *CCS Fitness, 288 F.3d at 1367, 62 USPQ2d at 1662-63* ("[A] claim term will not carry its ordinary meaning if the intrinsic evidence shows that the patentee distinguished that term from prior art on the basis of a particular [*1354] embodiment, expressly disclaimed subject matter, or described a particular embodiment as important to the invention."). If the claim term "therapeutically effective" encompasses the patient responses described in the specification, as it appears to us it does, [**110] then the Goldwasser study may constitute invalidating prior art under § 102(a) or § 103 even if he did not achieve his intended result. We therefore vacate the trial court's determination that Goldwasser cannot constitute prior art because the study was a failure. Resolution of the issue turns on the construction of the meaning of "therapeutically effective," which the trial court should have an opportunity to construe in the first instance under Markman principles. See *Bayer AG v. Biovail Corp., 279 F.3d 1340, 1349, 61 USPQ2d 1675, 1682 (Fed. Cir. 2002).* Accordingly, on remand, the court should construe this term and, in light of that construction, should determine whether Goldwasser invalidates any of the asserted patents under *35 U.S.C. § § 102*(a) or 103. n20

> n20 We note also that on remand when considering obviousness and anticipation issues relating to the *'080* and *'422* patents the district court should be cognizant of the rule that a claimed product shown to be present in the prior art cannot be rendered patentable solely by the addition of source or process limitations. *General Electric Co. v. Wabash Co., 304 U.S. 364, 373, 82 L. Ed. 1402, 58 S. Ct. 899, 1938 Dec. Comm'r Pat. 813 (1938); Cochrane v. Badische Anilin & Soda Fabrik, 111 U.S. 293, 311, 28 L. Ed. 433, 4 S. Ct. 455, 1884 Dec. Comm'r Pat. 230 (1884).*

[**111]

B

A second item of prior art germane to this appeal is United States Patent No. *4,377,513* ("Sugimoto"), issued in March 1983. Sugimoto discloses a process for producing human erythropoietin "characterized by multiplying human lymphoblastoid cells capable of producing human erythropoietin by transplanting said cells into a non-human warm-blooded animal body, or alternatively multiplying said cells by allowing said cells to multiply with a device by which the nutrient body fluid of a non-human warm-blooded animal is supplied to said cells, and allowing the cells multiplied by either of the above multiplication procedures to release human erythropoietin." Sugimoto, col. 1, lines 30-38. Given the similarity of Sugimoto's disclosure to the patents asserted by Amgen, TKT naturally raised Sugimoto as potentially invalidating prior art, even though Sugimoto had been before the examiner.

The district court concluded that Sugimoto was not prior art under *35 U.S.C. § 102*(a) because it was not proven to be enabled. *Amgen, 126 F. Supp. 2d at 108, 57 USPQ2d at 1476* ("In light of the intense competition that grew out of the race to make human EPO suitable [**112] for treatment of chronic anemia, one would imagine that if Sugimoto's invention were truly enabling, then he would have won that lucrative race."). On appeal, TKT argues that the trial court erred in placing on it the burden of proving enablement of Sugimoto, because United States patents -- even those only asserted as prior art in an invalidity defense -- are presumed enabled under *35 U.S.C. § 282.* We agree that prior art patents are presumed enabled, but under authority going beyond § 282.

[HN28] A claimed invention cannot be anticipated by a prior art reference if the allegedly anticipatory disclosures cited as prior art are not enabled. Long ago our predecessor court recognized that a non-enabled disclosure cannot be anticipatory (because it is not truly prior art) if that disclosure fails to "enable one of skill in the art to reduce the disclosed invention to practice." *In re Borst, 52 C.C.P.A. 1398, 345 F.2d 851, 855, 145 USPQ 554, 557 (C.C.P.A. 1962);* accord *In re Donohue, 766 F.2d at 533, 226 USPQ at 621.* Thus, the critical issue here is not whether Sugimoto [*1355] must be enabled, but rather whether it is the plaintiff or the defendant [**113] who bears the burden of proof with respect to that question.

On appeal, Amgen argues that there should be no presumption of enablement in this case because under § 282 courts only presume the claimed subject matter in a patent is enabled. Thus, Amgen argues, because only the unclaimed disclosures of Sugimoto are at issue here, no presumption of enablement should apply. This argument is not relevant, however, because, as reasoned below, we do not only rely on § 282 as the source for a

presumption. Instead, relying on our precedent, we hold a presumption arises that both the claimed and unclaimed disclosures in a prior art patent are enabled.

[HN29] In patent prosecution the examiner is entitled to reject application claims as anticipated by a prior art patent without conducting an inquiry into whether or not that patent is enabled or whether or not it is the claimed material (as opposed to the unclaimed disclosures) in that patent that are at issue. n21 *In re Sasse, 629 F.2d 675, 681, 207 USPQ 107, 111 (C.C.P.A. 1980)* ("When the PTO cited a disclosure which expressly anticipated the present invention . . . the burden was shifted to the applicant. He had to rebut the [**114] presumption of the operability of [the prior art patent] by a preponderance of the evidence." (citation omitted)). The applicant, however, can then overcome that rejection by proving that the relevant disclosures of the prior art patent are not enabled. Id. We hold that an accused infringer should be similarly entitled to have the district court presume the enablement of unclaimed (and claimed) material in a prior art patent defendant asserts against a plaintiff. Thus, a court cannot ignore an asserted prior art patent in evaluating a defense of invalidity for anticipation, just because the accused infringer has not proven it enabled. Like the applicant in ex parte prosecution, however, the patentee may argue that the relevant claimed or unclaimed disclosures of a prior art patent are not enabled and therefore are not pertinent prior art. If a patentee presents evidence of nonenablement that a trial court finds persuasive, the trial court must then exclude that particular prior art patent in any anticipation inquiry, for then the presumption has been overcome. n22 Therefore, it was Amgen who bore the burden of proving the nonenablement of Sugimoto before the district court. [**115] TKT did not bear a burden of proving enablement.

n21 Additionally, we think it unwise as a matter of policy to force district courts to conduct a mini-trial on the proper claim construction of a prior art patent every time an allegedly anticipating patent is challenged for lack of enablement. As we frequently revisit district courts' determinations in matters of claim construction and validity, we are certainly aware that such a task can occupy a great deal of a court's resources. In any event, because the presumption outlined here does not rely on § 282, we see no reason to impose these burdens on litigants and the district courts.

n22 We note that by logical extension, our reasoning here might also apply to prior art printed publications as well, but as Sugimoto is a patent we need not and do not so decide today.

Turning now to the district court's opinion, we think a fair reading is that the court, at least implicitly, put a burden of proving enablement of Sugimoto on TKT. The court began its analysis [**116] of Sugimoto by discussing evidence from Amgen and concluding "one would imagine that if Sugimoto's invention were truly enabling, then he would have won that lucrative race [to make human EPO suitable for treating anemia]." *Amgen, 126 F. Supp. 2d at 108, 57 USPQ2d at 1476.* Proceeding from that standpoint, the court analyzed whether [*1356] TKT's evidence was sufficient "to counter" this apparent conclusion that Sugimoto was not enabled. *Id. at 108-09, 57 USPQ2d at 1476.* Next, the court concluded its discussion of the enablement of Sugimoto by stating "TKT provided no evidence adequate to overcome the presumption that the Patent Office correctly rejected the contention that Sugimoto was an anticipating reference." *Id. at 109, 57 USPQ2d at 1477.* Importantly, only after apparently concluding that Sugimoto was not enabled did the district court discuss whether Sugimoto contained each and every limitation of any of Amgen's claims. The logical implication being that the court concluded that because TKT had not proven the enablement of Sugimoto, it could not anticipate any of Amgen's claims. In sum, we determine that ultimately, the district court [**117] placed the burden of proving the enablement of Sugimoto on TKT.

In addition, looking at the evidence Amgen did present, we cannot conclude the district court properly found Amgen had met any burden that the court did place on it. At trial Amgen's expert, Dr. Erslev, testified that "no one reported using Sugimoto's process to make a pharmaceutical composition of human EPO, nor has any patient ever been treated by any EPO produced by the Sugimoto procedure." *Id. at 108, 57 USPQ2d at 1476.* The mere fact that no one has so used the Sugimoto process is only minimally probative of non-enablement: a conclusion that no one could have used Sugimoto. Amgen also pointed out that Sugimoto was before the patent examiner during the prosecution of Amgen's patents. Id. While this was true, Sugimoto's non-enablement was only one of several arguments Amgen presented to overcome a rejection during prosecution and the examiner did not state his agreement with this position when he allowed the patent. Because we cannot assume the acceptance of every argument presented during prosecution, the mere fact this argument was made is also only minimally probative of the enablement [**118] of Sugimoto. In sum, the evidence presented by Amgen was insufficient to meet the burden Amgen apparently was assigned.

We must therefore conclude that to the extent it placed a burden on TKT the district court committed error. However, we hold this error to be, for the most part, harmless. After analyzing enablement and apparently finding the relevant unclaimed disclosures of Sugimoto nonenabled, the court nevertheless conducted a full anticipation analysis. Indeed, the district court performed a detailed analysis of each piece of anticipating prior art -- including Sugimoto -- asserted against each of Amgen's claims. *Id. at 109-10, 57 USPQ2d at 1477.* From this analysis the court found that "none of the cited references disclose [sic] each and every limitation of any of Amgen's individual claims." *Id. at 109, 57 USPQ2d at 1477.* It does not appear that TKT has argued this alternative finding was clear error. However, we do not rest on waiver, but affirm the district court's finding that Sugimoto does not anticipate any asserted claims of the *'080*, *'349*, or *'698* patents because from our review of the evidence and the subsidiary finding of the court, [**119] it was not clear error to find in each claim one or more limitations not disclosed in Sugimoto. But given our earlier holdings, we must vacate and remand the finding that Sugimoto does not anticipate claim 1 of the *'422* patent. On remand, the district court should consider whether claim 1 of the *'422* patent is novel over Sugimoto in light of the court's new definition of "therapeutically effective" and while mindful of the principle that source limitations cannot impart novelty to old compositions.

Our review is not yet finished, however, because it is apparent from the [*1357] district court's opinion that TKT relied upon Sugimoto to assert invalidity of the patents in suit under both § 102 and § 103. In its obviousness inquiry, the district court disregarded Sugimoto because it concluded it was not enabled. It recognized, however, the important and potentially dispositive role that Sugimoto would have otherwise played in the obviousness analysis:

> Had the court concluded otherwise [i.e., that Sugimoto was enabled], the Sugimoto patent would go a long way toward proving TKT's obviousness defense. As explained above, Sugimoto disclosed EPO-producing fused cells and advised [**120] that (1) conventional techniques can be utilized to achieve purification and (2) the human EPO produced thereby can be used in pharmaceutical compositions for the treatment of anemia. Thus, the patent itself suggested combining its invention with prior art sources relating to both purification and therapeutic delivery. Provided that one of ordinary skill in the art could actually make the EPO-producing cells described in the Sugimoto patent, a point on which TKT failed to persuade this court, such a combination of prior art materials might render invalid the pharmaceutical composition claims of the *'933*, *'080*, and *'422* patents.

Id. at 114 n.29, 57 USPQ2d at 1480 n.29. [HN30] Under § 103, however, a reference need not be enabled; it qualifies as a prior art, regardless, for whatever is disclosed therein. See *Symbol Tech., Inc. v. Opticon, Inc., 935 F.2d 1569, 1578, 19 USPQ2d 1241, 1247 (Fed. Cir. 1991); Reading & Bates Constr. Co. v. Baker Energy, 748 F.2d 645, 652, 223 USPQ 1168, 1173 (Fed. Cir. 1984).* Therefore, the district court's obviousness holdings with respect to Sugimoto are vacated and remanded. On remand, the district court [**121] should reconsider obviousness with respect to Sugimoto, but should do so without reference to whether Sugimoto is enabled, as enablement of the prior art is not a requirement to prove invalidity under § 103.

V

The last issue on appeal is inequitable conduct. TKT raised before the district court essentially three instances of allegedly inequitable activities by the patentee: withholding crucial details regarding the Goldwasser study; withholding certain results of its own experiments that undermined the validity of the *'933* patent; and failing to disclose to the Patent and Trademark Office the existence of this litigation. The district court found that TKT had not proven inequitable conduct by clear and convincing evidence, and we have not been persuaded on appeal that a contrary result is compelled. In reaching this conclusion, we need look no further than the district court's determination that TKT's case was doomed because it was bereft of evidence of intentional deception:

> TKT has failed to produce any persuasive evidence that causes the Court to doubt the integrity of the individuals who bore the duty of shepherding the Amgen patent applications through the Patent and [**122] Trademark Office, [so] its charge of inequitable conduct utterly fails . . . . TKT has failed to prove by clear and convincing evidence that this [experimental] data was material or that it was withheld with intent to deceive . . . . [And] TKT has not even begun to demonstrate that Amgen representatives possessed an intent to deceive the [PTO] in failing to provide specific notification

> regarding this litigation . . . . In summary, TKT's proof of inequitable conduct with respect to each of these charges falls short of the mark. Although the directness of Amgen's disclosures varies depending on the particular piece of disputed information, one truth remains the same throughout: Amgen's [*1358] representatives never intended to deceive the Patent Office. Consequently, a finding of inequitable conduct would be error and the Court does not so find on the complete record.

Id. at 141, 145, 147, 57 USPQ2d at 1500, 1504, 1505.

[HN31] A patent applicant commits inequitable conduct when, during prosecution of the application, he makes an affirmative representation of a material fact, fails to disclose material information, or submits false material information, and does so with the intent [**123] to deceive. *Refac Int'l, Ltd. v. Lotus Dev. Corp., 81 F.3d 1576, 1581, 38 USPQ2d 1665, 1669 (Fed. Cir. 1996).* As a general principle, materiality and intent are balanced -- a lesser quantum of evidence of intent is necessary when the omission or misrepresentation is highly material, and vice versa. See, e.g., *GFI, Inc. v. Franklin Corp., 265 F.3d 1268, 1273, 60 USPQ2d 1141, 1143 (Fed. Cir. 2001).* At the same time, however, there must be some threshold showing of intent to be balanced; we will not find inequitable conduct on an evidentiary record that is completely devoid of evidence of the patentee's intent to deceive the PTO. See *Allen Eng'g Corp. v. Bartell Indus., Inc., 299 F.3d 1336, 2002 U.S. App. LEXIS 15418,* at *33 (Fed. Cir. 2002) ("Materiality does not presume intent, which is a separate and essential component of inequitable conduct." (quoting *Allen Organ Co. v. Kimball Int'l, Inc., 839 F.2d 1556, 1567, 5 USPQ2d 1769, 1778 (Fed. Cir. 1988))).*

Here, the district court determined that there was no evidence of intent to deceive, and TKT has directed us to none on appeal. Thus, to conclude [**124] the Amgen patents are unenforceable -- as TKT requests -- we must conclude (1) that the district court clearly erred by failing to find the minimal requisite intent to deceive, and (2) that it abused its discretion in weighing the degree of materiality against the degree of deceptive intent and by not then rendering the patents unenforceable. On the record before us, we decline to do so.

CONCLUSION

We summarize our decision as follows. Affirmed are: the district court's claim construction; its finding that all of the patents in suit are enforceable; its finding that the *'933* patent is invalid; and its finding that the *'349* (product claims only) and the *'422* patents are infringed. We vacate: its finding that the *'933* patent was not infringed; several of its validity findings with respect to the *'080*, the *'349*, the *'422*, and the *'698* patents; and its infringement findings with respect to the *'698* patent and *'349* patent claim 7. On remand, the district court should: construe the claim term "therapeutically effective" and then reconsider validity under § § 102 and 103 in view of Goldwasser; reconsider validity of all asserted claims under § 103 and claim 1 of the *'422* patent under § [**125] 102 in view of Sugimoto, with Amgen bearing the burden of proof on its non-enablement (for § 102 purposes only); reassess infringement of the accused method by comparing it solely to the limitations of each of the asserted method claims; and reevaluate its finding of infringement under the doctrine of equivalents of the *'080* patent, focusing on the application of prosecution history estoppel.

AFFIRMED IN PART, VACATED IN PART, REMANDED.

No costs.

DISSENTBY: CLEVENGER (In Part)

DISSENT: CLEVENGER, Circuit Judge, dissenting in part.

I join my colleagues' thorough opinion in all respects save one, albeit significant, exception. Because the claims lack meaningful limitations on the structure of the erythropoietin-producing cells, I cannot [*1359] agree that the district court should have abstained from inquiring fully whether the claims were suspect under the enablement and written description provisions of *35 U.S.C. § 112,* P 1.

As described by the specifications of the patents in suit, Amgen in 1984 cloned and sequenced the DNA encoding human erythropoietin (EPO). Amgen then showed that by introducing the cloned EPO DNA (linked to a promoter sequence) into mammalian [**126] cells, those cells could be engineered to express high levels of functional human EPO protein. The parties refer to this as "exogenous DNA" expression of EPO. Amgen obtained several patents that cover the use and manipulation of cloned EPO DNA, and these patents, battle-tested through litigation, have been the foundation of Amgen's successful business of manufacturing and selling recombinant EPO. But these patents are not in suit here, and TKT's method for producing EPO does not rely upon manipulation of cloned EPO DNA or "exogenous DNA" expression technology.

The claims in suit here contain no significant limitations as to how the recombinant EPO is expressed,

or as to the structure of the EPO-producing cells, so long as the EPO is "non-naturally occurring" or produced in "vertebrate cells." The central question in this case is therefore whether Amgen's disclosure of one means of producing synthetic EPO in mammalian cells, namely exogenous DNA expression, entitles it to claim all EPO produced by mammalian cells in culture, or all cultured vertebrate cells that produce EPO. I think this is a question of some importance. Yet it is a question that the district court simply [**127] refused to consider. Although the district court admitted that Amgen's disclosure was limited to exogenous DNA expression, the district court quite clearly and explicitly refused to decide whether the absence of any exogenous DNA limitations rendered the asserted claims vulnerable to the enablement challenge mounted by TKT under section 112. According to the district court, because the asserted claims were to "compositions" rather than "processes," "the specification need teach only one mode of making and using a claimed composition." *Amgen Inc. v Hoescht Marion Roussel, Inc., 126 F. Supp. 2d 69, 160, 57 USPQ2d 1449, 1515 (D. Mass. 2001).* See also *id. at 160, 164 n.57, 57 USPQ2d at 1516, 1518 n.57.* Likewise, the district court refused to inquire whether the absence of limitations on the means of EPO expression raised questions of compliance with the written description requirement, holding that such an inquiry was irrelevant to composition claims. *Id. at 150-51, 57 USPQ2d at 1508.*

With respect to the *'080* and *'422* patents, which claim "non-naturally occurring" EPO and EPO "purified from mammalian cells grown in culture," the majority, [**128] like the district court, essentially passes over the question of whether these limitations--which are essential for patentability of the claims--raise issues of compliance with the enablement and written description requirements of section 112. The majority holds that patentees are free to decorate their composition claims with source and process limitations without any concern for whether the full scope of those limitations is enabled or described, and that these requirements of section 112 are waived so long as the patentee succeeds in characterizing its claims as "product" claims. Competent patent attorneys should be quick to take advantage of the majority's broad exemption from the disclosure requirements by the appropriate phraseology. Rather than endorse the district court's elevation of form over substance, I would vacate its decision on these issues regarding the *'080* and *'422* patents, and remand for further consideration [*1360] in light of the vast scope of the claims in suit for which there appears to be insufficient enabling disclosure or written description.

With particular reference to the *'349* patent, which claims not EPO polypeptides but the cells that produce them, I think [**129] the district court's abstention from scrutiny under section 112 is even more patent error. The majority focuses on the district court's findings that the invention could readily be practiced in mammalian or vertebrate cells other than the hamster and monkey cells taught by the specification. I agree that TKT has not shown error in these findings. But, as it did for the EPO claims, the district court simply refused to consider whether the absence of any exogenous DNA limitations raised enablement issues, "because Amgen is only required to enable skilled artisans to make its claimed product by only one method . . . ." *Id. at 164 n.57, 57 USPQ2d at 1518 n.57.* For the EPO-secreting cells, the absence of an exogenous DNA limitation is not a failure to limit how the product is made, but a failure to limit the structure of the claimed product itself. A cell, as employed in the patents in suit, is nothing more than a biological machine for making EPO. Even in more predictable arts, one who is first to make a machine is not entitled as a matter of law to claim any or all machines so long as they perform the same function. I would think it uncontroversial that even one who is [**130] first to make polymer X or alloy Y cannot obtain a claim as broad as "A machine that makes polymer X," or "A process that yields alloy Y," without reciting additional limitations that define the structure of the claimed machine or the steps necessary to carry out the claimed process.

Yet that is exactly what the district court and the majority allow the *'349* patent to achieve. It claims any or all cultured vertebrate cells that can secrete a defined amount of EPO, with only a single limitation on their structure: that they "comprise non-human DNA sequences which control transcription of DNA encoding human erythropoietin," or that they "comprise transcription control DNA sequences, other than human erythropoietin transcription control sequences, for production of human erythropoietin." This is little more precise than a recitation of "A machine that makes polymer X, wherein the machine comprises means for controlling how much polymer X is made." The specification teaches only a single means by which the use of a transcription control sequence can coax a vertebrate cell to secrete EPO: by transforming that cell with an exogenous expression vector on which the transcription control sequence [**131] is linked to cloned EPO DNA. Yet the claims leave this essential aspect of the invention undefined. It is black-letter law that claims failing to recite a necessary element of the invention fail for lack of an enabling disclosure, *In re Mayhew, 527 F.2d 1229, 1233, 188 USPQ 356, 358 (CCPA 1976),* and that disclosure of one or two species may not enable a broad genus under these circumstances. *In re Vaeck, 947 F.2d 488, 496, 20 USPQ2d 1438, 1444-45 (Fed. Cir. 1991).* At the very least, the absence of

structural limitations in the *'349* patent raises questions of its enablement, and I cannot agree that the district court chose correctly by ignoring those questions altogether. We should vacate the district court's judgment that the *'349* patent passes enablement muster, and require the court to apply the correct law to the plain facts.

I must also disagree with the majority that the district court's approach was faithful to this court's articulation of the written description requirement of section 112, as expressed in *Regents of the University of California v. Eli Lilly & Co., 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997)* [*1361] and *Gentry Gallery, Inc. v. Berkline Corp., 134 F.3d 1473, 45 USPQ2d 1498 (Fed. Cir. 1998).* [**132] Eli Lilly articulated two principles of the written description requirement: that in haec verba description of broadly described generic subject matter may not suffice to describe the subject matter of that particular claim, *119 F.3d at 1567, 43 USPQ2d at 1404-05,* and that disclosure of a species may not suffice to describe a genus, *id. at 1568-69, 43 USPQ2d at 1405-06.* The district court followed neither of these principles here, and the majority, dismissing Eli Lilly on the grounds that no undisclosed DNA molecule appears in this case, verges on confining Eli Lilly to its facts.

Nor am I convinced that the district court's approach was faithful to Gentry Gallery. In Gentry, only those claims that included limitations such as "wherein the control means are located on the console" satisfied the written description requirement. Because the specification failed to disclose any location for the controls other than on the console, those claims that lacked such limitations were invalid under § 112, P 1. *134 F.3d at 1479-80, 45 USPQ2d at 1503-04.* The question here is similar: whether the claims fail the written description [**133] requirement for lack of "exogenous DNA" limitations, because the specification discloses only the exogenous DNA technology that was state of the art in 1984.

Even if we ignore the patents' statement that the claimed EPO molecules are "uniquely characterized by being the product of . . . expression . . . of exogenous DNA sequences" (which of course we cannot), I think the parallels between this case and Gentry Gallery are inescapable. The invalid claims in Gentry recited elements that could readily be found in the text of the specification (a couch, controls, a console), but those claims nonetheless failed the written description requirement because they included no limitations on how those elements were arranged. Likewise, the *'349* claims--for which I think it must be conceded that structure of the EPO-secreting cell is a relevant question--recite particular elements found in the specification (cells, non-human control sequences, EPO-coding DNA), but do not include limitations on the arrangement of those elements, e.g. that the non-human control sequences and coding DNA are present on an exogenous expression vector in the cell. I agree that as a matter of claim interpretation [**134] there is no justification for importing an "exogenous DNA" limitation into the claims. But the absence of such limitations must weigh heavily in the section 112 inquiry, else we hold that claims become more resistant to written description challenges the more broadly drafted they are.

While I share my colleagues' admiration for the considerable efforts of the district court in this complicated case, I cannot share their faith that the district court properly and conscientiously applied Eli Lilly and Gentry Gallery, when the district court's opinion is completely devoid of reference either to those cases or to the principles they espouse. If the district court did not focus on the correct law to be applied, then its factual findings merit no deference, and the correct remedy for this omission is to vacate the district court's judgment on this point and remand for further consideration. Our precedent has little value if the district courts may overlook its certain pertinence, if not its plain applicability.

Example 18: <u>Process claim where the novelty is in the method steps.</u>

Specification: The specification teaches a method for producing proteins using mitochondria from the fungus *Neurospora crassa*. In the method, mitochondria are isolated from this fungus and transformed with a mitochondrial expression vector which comprises a nucleic acid encoding a protein of interest. The protein is subsequently expressed, the mitochondria is lysed, and the protein is isolated. The specification exemplifies the expression of β-galactosidase using the claimed method using a cytochrome oxidase promoter.

Claim:

1. A method of producing a protein of interest comprising;

obtaining *Neurospora crassa* mitochondria,

transforming said mitochondria with a expression vector comprising a nucleic acid that encodes said protein of interest,

expressing said protein in said mitochondria, and

recovering said protein of interest.

Analysis:

A review of the specification reveals that *Neurospora crassa* mitochondrial gene expression is essential to the function/operation of the claimed invention. A particular nucleic acid is not essential to the claimed invention.

A search of the prior art reveals that the claimed method of expression in *Neurospora crassa* is novel and unobvious.

The claim is drawn to a genus, i.e., any of a variety of methods that can be used for expressing protein in the mitochondria.

There is actual reduction to practice of a single embodiment, i.e., the expression of β-galactosidase.

The art indicates that there is no substantial variation within the genus because there are a limited number of ways to practice the process steps of the claimed invention.

The single embodiment is representative of the genus based on the disclosure of *Neurospora crassa* mitochondria as a gene expression system, considered along with the level of skill and knowledge in the gene expression art. One of skill in the art would recognize that applicant was in possession of all of the various expression methods necessary to practice the claimed invention.

Conclusion:

The claimed invention is adequately described.

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☑ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☑ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.